UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

DEPARTAMENTO DE BIOLOGÍA

LAS ENZIMAS 9-LIPOXIGENASAS Y EL ETILENO REGULAN LA RESPUESTA DE ESTRÉS OXIDATIVO DURANTE LA ACTIVACIÓN DE LA INMUNIDAD VEGETAL

TESIS DOCTORAL

Miguel Ángel López Carrasco

Madrid, 2012

LAS ENZIMAS 9-LIPOXIGENASAS Y EL ETILENO REGULAN LA RESPUESTA DE ESTRÉS OXIDATIVO DURANTE LA ACTIVACIÓN DE LA INMUNIDAD VEGETAL

Memoria presentada para optar al grado de

Doctor en Ciencias,

por

Miguel Ángel López Carrasco

Madrid, Mayo 2012

VºBº del Director de Tesis

Dra. Carmen Castresana Fernández

VºBº del Tutor de Tesis

Dra. Francisca Fernández del Campo

El Doctorando

Miguel Ángel López Carrasco

“Casi todo lo que realice será insignificante, pero es muy importante que lo haga”

Mahatma Gandhi

RECONOCIMIENTOS

Este trabajo ha sido realizado en el Centro Nacional de Biotecnología, perteneciente al Consejo Superior de Investigaciones Científicas

Quiero expresar mi más sincero agradecimiento a todas las personas que han colaborado de alguna manera en la realización de este trabajo.

En primer lugar, a mi directora de Tesis la Prof. Carmen Castresana Fernández, por la inigualable labor de dirección realizada y por la confianza depositada en mí.

Deseo agradecer a mi tutora de Tesis, Dra. Francisca Fernández del Campo su amabilidad y colaboración en la realización de este trabajo

Al Prof. Matts Hamberg por facilitarnos las oxilipinas usadas en este trabajo y por la realización de los análisis de GC/MS.

Al Prof. Roberto Solano por cedernos semillas del mutante *ein2-5.*

Al Prof. J.R Ecker por cedernos las semillas de plantas transgénicas *35S::EIN3:GFP.*

Al Prof. J. Alonso por cedernos las semillas de plantas transgénicas *EBS::GUS.*

Al departamento de Genética Molecular de Plantas del Centro Nacional de Biotecnología en especial al Servicio de Invernadero y Unidad de Cultivo *in vitro*, cuya labor ha facilitado enormemente la realización de este trabajo.

Al departamento de Imagen y Fotografía del Centro Nacional de Biotecnología por la realización de la fotos que aparecen en la tesis.

Al servicio de Genómica por los análisis transcriptómicos presentados en este trabajo.

A los departamentos de Personal y Administración por facilitar y solucionar todos los trámites y dudas administrativas.

A todos los miembros del laboratorio 315 por su inigualable disposición a la colaboración y sin la cual hubiera sido imposible la consecución este trabajo.

ABREVIATURAS

ABREVIATURAS

ABA: ácido abscísico

ACC: 1-aminocyclopropane-1-carboxylic acid

ADN: ácido desoxirribonucleico

ADNc: ADN complementario

AOS: aleno óxido sintasa

ARN: ácido ribonucleico

***avr*:** gen de avirulencia

BAC: *bacterial artificial chromosome*

BL: brasinoesteroides

BLAST: *basic local alignment research tool*

BrEt: bromuro de etidio

CAPS: *cleaved amplified polymorphic sequences*

CK: citoquininas

Col-0: Columbia-0

CTR1: *constitutive triple response 1*

DAB: *Diaminobenzidina*

DES: divinil éter sintasa

DO: densidad óptica

DOX: dioxigenasa

EAS: epoxi alcohol sintasa

EBS: *ethylene binding site*

EDTA: ácido etilen-diamino-tetra-acético

EIN2: *ethylene insensitive 2*

EMS: metano sulfonato de etilo

ET: etileno

ETI: effector-triggered immunity

ETO1: *ethylene overproduction 1*

GA: giberelinas

GO: *Gene Ontology*

GUS: *β-glucuronidasa*

H_2O_2: peróxido de hidrógeno

HPL: hidroperóxido liasa

HPLC: *High Performance Liquid Chromatography*

HR: respuesta hipersensible

JA: ácido jasmónico

Kb: kilobase

KB: medio King´s B

LB: medio Luria-Bertani

LOX: lipoxigenasa

mA: miliamperios

MAMP: *microbial-asociated molecular pattern*

$MgCl_2$: cloruro de magnesio

MS: medio Murashige y Skoog

NCBI: *national center of biotechnology information*

NBT: nitroazul de tetrazolio

NO: óxido nítrico

***noxy*:** non responding to oxylipin

$^1O_2$: singlete de oxígeno

O_2^-: ión superóxido

OH^-: radical hidroxilo

PAMP: *pathogen-associated molecular pattern*

PCR: reacción en cadena de la polimerasa

PDF: defensina

PR: *pathogenesis related protein*

PRR: *pattern recognition pattern*

***Pst*:** *Pseudomonas syringae* pv *tomato*

PTI: *PAMPs-triggered immunity*

PXG: peroxigenasas

r.p.m: revoluciones por minuto

RB: rosa de bengala

***R*:** gen de resistencia

ROS: especies reactivas de oxígeno

RT-PCR: transcripción reversa seguida de PCR

SA: ácido salicílico

SAR: *systemic acquired resistance*

SDS: dodecil sulfato sódico

SNP: *Single nucleotide polymorphism*

SSC: solución NaCl 150 mM, citrato sódico 15 mM

SSLP: *simple sequence lenght polimorphism*

TAIR: *the Arabidopsis information resource*

TBE: tampón Tris-ácido bórico 90 mM, NA_2-EDTA 2 mM pH8

T-ADN: ADN transferente

Tris: tris-(hidroximetil)-aminometano

ufc: unidades formadoras de colonias

ÍNDICE

ÍNDICE

RESUMEN/ABSTRACT

RESUMEN

En el presente trabajo nos propusimos examinar la participación de los enzimas 9-LOX, y por tanto de las oxilipinas generadas a través de esta ruta biosintética, en la respuesta de las plantas frente a la infección de bacterias hemibiotrofas del género *Pseudomonas*. A este objeto, diseñamos una estrategia genética dirigida a generar y caracterizar plantas mutantes carentes de actividad 9-LOX (*lox1 lox5*), así como a caracterizar los mutantes *noxy6 y noxy22* (*non-responding to oxylipins*) alterados en la señalización activada por la acción del ácido 9-hidroxitrienoico (9-HOT), compuesto derivado de las 9-LOX, cuya participación en la defensa de la planta se concluye de los resultados que demostraban su acumulación en respuesta a una infección patogénica, así como de su acción como señal activadora de respuestas de defensa.

Los resultados de este trabajo han permitido identificar la presencia en *Arabidopsis* de dos genes, *LOX1* y *LOX5*, que codifican enzimas con actividad 9-LOX, y generar dobles mutantes *lox1 lox5* carentes de dicha actividad. El análisis de los mutantes *lox1 lox5* ha puesto de manifiesto que la falta de actividad 9-LOX aumenta la susceptibilidad de las plantas frente a la infección de bacterias

virulentas y, por tanto, que la actividad 9-LOX contribuye de forma positiva a la inducción de la respuesta de defensa.

De forma paralela, la caracterización de los mutantes *noxy6* y *noxy22*, insensibles a la acción del 9-HOT, ha permitido localizar dichas mutaciones en los genes *CTR1* (CONSTITUTIVE ETHYLENE RESPONSE1) y *ETO1* (ETHYLENE-OVERPRODUCER1), que actúan, respectivamente, como reguladores negativos de la respuesta a etileno (ET). De acuerdo a estos resultados, la caracterización de los mutantes *noxy*, renombrados *ctr1-15* y *eto1-14*, ha puesto de manifiesto que la activación constitutiva de la respuesta a etileno interfiere con la acción del 9-HOT. Además, el estudio detallado de esta interacción ha revelado que el 9-HOT impide la activación de la respuesta a etileno y por tanto, que dichas señales, ET y 9-HOT ejercen un efecto antagónico recíproco.

El estudio de la respuesta del mutante *eto1-14* a la infección de bacterias patógenas pone de manifiesto que, la producción constitutiva de etileno interfiere con la activación de la defensa vegetal y que en este mutante, al igual que en el mutante *lox1 lox5*, el aumento de la susceptibilidad está asociado a una alteración en la regulación del estrés oxidativo producido como consecuencia de la infección.

El análisis de la respuesta de las plantas *lox1 lox5* y *eto1-14* (insensibles a 9-HOT) a distintos tipos de estrés oxidativo, ha revelado la participación de los enzimas 9-LOX y del ET en la respuesta de la planta a la generación de oxígeno singlete, en donde la falta de actividad 9-LOX (en los mutantes *lox1 lox5*), o el defecto en la señalización de la respuesta a su derivado, 9-HOT (mutante *eto1-14*), conlleva un aumento del daño celular causado por este tipo de especies reactivas. Finalmente, el análisis transcripcional de la respuesta de los mutantes *lox1 lox5* y *eto1-14*, a la generación de oxígeno singlete, permite confirmar estos resultados y demostrar la acción de estas rutas de señalización en la regulación del estrés oxidativo que ocurre durante la infección de patógenos, cuyo control es esencial para la activación de una respuesta de defensa eficaz con capacidad para limitar el crecimiento de patógenos hemibiotrofos.

ABSTRACT

Enzymatic synthesis of oxylipins is initiated by incorporation of oxygen into a fatty acid molecule, catalyzed by the activities of 9- and 13-lipoxygenases or α-dioxygenases, initiating the biosynthesis of an extensive family of metabolites. Much attention has been paid to the biosynthetic pathway initiated by 13-lipoxygenases (13-LOX) which plays critical in plant defense responses. However the participation of oxylipins produced by 9-LOX and α-DOX pathways, and the role of non-enzymatically generated oxylipins in regulating plant defense responses remain poorly understood.

Here we proposed to study the role of 9-LOX enzymes, and the oxylipins generated through this pathway in plant response to *Pseudomonas* infection. For this purpose, we made use of a double mutant, *lox1 lox5*, that lacks 9-LOX activity, and of two 9(S)-hydroxy-10,12,15-octadecatrienoic acid (9-HOT) insensitive mutants, *noxy6* and *noxy22* (non-responding to oxylipins). A number of studies have demonstrated that 9-HOT is accumulated during pathogen infection, and their action as a signal activating defense responses.

Double mutants that lack 9-LOXs activity were generated. This mutant, *lox1 lox5*, contains a T-DNA insertion in each of the two *9-LOXs* genes of *Arabidopsis*. Analyses of the response to the hemibiotrophic bacterial strains *Pst* DC3000 revealed that lack of 9-LOX activity enhance bacterial susceptibility, and therefore

we demonstrated that oxylipins produced by 9-LOXs activity participates in the defense of plants against virulent bacteria.

Map-based cloning showed that *noxy6* and *noxy22* have a CTR1 and ETO1 gene mutations, and were renamed as *ctr1-15* and *eto1-14*, respectively. These proteins, CTR1 and ETO1, act as negative regulators of ethylene response. As a consequence of *ctr1-15* and *eto1-14* mutations, the ET pathway was constitutively activated in these plants and according to these results constitutive activation of ethylene response interferes with 9-HOT signaling. In addition to the inhibitory effect of ET on 9-HOT signaling, a reciprocal interference was also found, indicating that the antagonistic action of ET and 9-HOT is bi-directional.

Analyses of the response to the hemibiotrophic bacterial strains *Pst* DC3000 *avr*Rpm1 (avirulent) and *Pst* DC3000 (virulent) revealed higher bacterial growth rates *eto1-14* mutants. These results indicate that constitutive ET production decreased plant defense response and in this mutant, as in the mutant *lox1 lox5*, increased susceptibility is associated with an alteration in the regulation of ROS.

The analysis of *eto1-14* and *lox1 lox5* response to different types of oxidative stress, has revealed the involvement of 9-LOX enzymes and ET in the control of plant response to singlet oxygen generation, where the lack of 9-LOX activity (in the mutant *lox1 lox5*), or defect in the signaling response to its derivative, 9-HOT (mutant *eto1-14*), leads to an increase cell damage caused by these reactive species. Finally, transcriptional analyses of wild-type plants, the *lox1 lox5* mutant (lacking 9-LOX activity) and the *eto1-14* mutant (disrupting 9-HOT signaling) after application of rose bengale, a generator of singlet oxygen, confirm these results and demonstrate the involvement of these signaling pathways in the regulation of oxidative stress that occurs during pathogen infection, in which the production of ROS must be tightly regulated to achieve full resistance and plant survival.

INTRODUCCIÓN

INTRODUCCIÓN

1. LA DEFENSA VEGETAL

Las plantas son organismos sésiles, que necesitan coordinar los programas de desarrollo que dirigen su crecimiento, con aquellos que facilitan su adaptación a condiciones ambientales variables. En este proceso de coordinación, las plantas utilizan herramientas diversas entre las que la acción de numerosos metabolitos, y en particular de las hormonas vegetales, juega un papel esencial. Los procesos de reprogramación celular activados por estos metabolitos, se establecen a través de rutas de transducción específicas que, además, interaccionan entre sí formando redes complejas de señalización. De esta manera, la respuesta de una planta a un estímulo cualquiera, exógeno o endógeno, no será nunca el resultado de la activación de una única ruta de transducción, sino, de un proceso de coordinación entre distintas rutas de señalización.

Un ejemplo relevante de esta coordinación lo constituye la activación de una respuesta de defensa frente a la infección de microorganismos patógenos, en la que las plantas tienen que modificar su metabolismo para dirigir parte de sus recursos energéticos a la generación de una gran cantidad de productos de defensa y, en donde, por tanto, la coordinación entre los programas de crecimiento, reproducción y defensa, es esencial para la supervivencia de la planta.

A lo largo de su evolución las plantas han desarrollado un complejo sistema inmunológico que les permite reconocer la presencia de microorganismos patógenos, y responder induciendo una respuesta de defensa dirigida a evitar la entrada y el crecimiento de los patógenos en la planta. Los estudios dirigidos a conocer los mecanismos de defensa vegetal, han permitido identificar los componentes celulares que participan en la percepción de los patógenos, así como en la generación y la transmisión de las señales que activan la producción de los compuestos de defensa. Estos estudios han puesto de manifiesto la participación del ácido salicílico (SA), los jasmonatos (JAS) y el etileno (ET), como las hormonas de defensa vegetal cuya producción y señalización es esencial para la activación del sistema inmune de la planta (Robert-Seilaniantz y col., 2007).

Estudios recientes demuestran que, además de las hormonas implicadas en la defensa (SA, JAS y ET), otras oxilipinas distintas del JA, y hormonas como los brasinoesteroides (BL), las auxinas, las giberelinas (GA), las citoquininas (CK) y el ácido abscísico (ABA), juegan un papel fundamental en la respuesta de la planta frente al ataque de patógenos. La acción de estas hormonas no estaría dirigida a limitar el crecimiento del patógeno, sino a regular procesos críticos para la supervivencia de la planta tales como, la redistribución de recursos energéticos, el control de la muerte celular, la regulación del estrés por hídrico asociado a la infección, etc., (Melotto y col., 2006; Robert-Seilaniantz y col., 2007; López y col., 2008; Navarro y col., 2008). Las interacciones entre estas rutas de señalización a través de redes complejas de señalización, representa

una importante ventaja adaptativa para las plantas, que les permite regular de forma precisa la respuesta frente a distintos tipos de patógenos.

Sin embargo, este sistema de regulación es, con frecuencia, utilizado por los patógenos para interferir con los mecanismos de defensa de la planta. De hecho, los patógenos han desarrollado la capacidad de producir hormonas vegetales y compuestos funcionalmente homólogos a éstas, que les permiten manipular la homeostasis hormonal de la planta. Además, los patógenos actúan modificando las rutas de señalización de la planta a través de los llamados efectores que, tras su liberación a la célula vegetal, poseen la capacidad de modular y reprogramar el metabolismo de la planta y redirigirlo para favorecer su desarrollo e interferir con la activación de la defensa.

1.1. ACTIVACIÓN DE LA INMUNIDAD VEGETAL Y LAS INTERACCIONES ENTRE LAS VÍAS DE SEÑALIZACIÓN QUE INDUCEN LA DEFENSA

Las plantas han desarrollado distintos sistemas de reconocimiento para detectar la presencia de patógenos, que actúan a distintos niveles activando respuestas de defensa complejas, que en su conjunto se engloban bajo el nombre de sistema de inmunidad vegetal.

Una vez en la planta, los patógenos encuentran un primer tipo de receptores, denominados PRR (del inglés, pattern recognition receptor) que reconocen moléculas del patógeno comunes a distintos microorganismos, denominadas MAMPs (del inglés, microbial-associated molecular pattern). Los receptores PRR se encuentran mayoritariamente localizados en la superficie de la célula vegetal, y poseen un dominio transmembrana y un dominio extracelular que participa en el reconocimiento. Además, algunos PRRs poseen un dominio kinasa intracelular que participa en la transmisión de la señalización (Lehti-Shiu y col., 2009).

En respuesta a este reconocimiento la plantas inducen una primera barrera de defensa no específica, denominada resistencia basal o MTI (del ingles, MAMP triggered immunity), que en muchas ocasiones permite controlar la

infección de la planta. No obstante, y ya que esto no ocurre en todos los casos, las plantas han desarrollado un segundo nivel de reconocimiento que activa una respuesta de defensa más eficaz e intensa, denominada resistencia inducida o ETI (del inglés, efector triggered immunity). Este segundo nivel de reconocimiento, es altamente específico y se establece mediante la interacción de los productos de los genes de resistencia de la planta (*R*), localizados fundamentalmente en el interior de la célula, con moléculas específicas del patógeno, denominadas efectores, que se liberan al interior de la célula vegetal durante la infección. La activación de la resistencia inducida está frecuentemente acompañada de la activación de un proceso de muerte celular local, en los puntos de infección, que da lugar a la formación de lesiones necróticas y se conoce como reacción hipersensible o HR (Jones y Dangl, 2006; Rosebrook y col., 2007). Cabe mencionar, que la reacción hipersensible se induce preferentemente en respuesta a la infección de patógenos biotrofos. De hecho, y dado el modo de vida de los patógenos necrotrofos, la activación de una reacción hipersensible podría conducir a un aumento de la susceptibilidad de la planta frente a este tipo de patógenos.

Las respuestas desencadenadas en los dos niveles de resistencia descritos, basal e inducida, son muy similares (Tsuda y col., 2010; Thomma y col., 2011; Qi y col., 2011) aunque existen diferencias entre ellas en relación a los primeros procesos de señalización que dirigen la activación de estas dos barreras inmunitarias (Zhang y Zhou, 2010; Zhang y col., 2010; Schwessinger y Zipfel, 2008). En general, estas respuestas implican cambios en el flujo de iones a través de la membrana plasmática, la generación de especies reactivas de oxígeno (ROS), la producción del óxido nítrico (NO), la deposición de calosa, la activación de proteínas quinasas, y la activación de la transcripción de numerosos genes de defensa cuyos productos de expresión contribuirán a limitar la invasión y el desarrollo del patógeno en la planta. (Alvarez y col. 1998; Gechev y col. 2006; Lehti-Shiu y col. 2009)

1.2. PAPEL DE LAS ESPECIES REACTIVAS DE OXÍGENO EN LA RESPUESTA DE DEFENSA

La producción de especies reactivas de oxígeno (ROS), como consecuencia del denominado estallido oxidativo, juega un papel clave en la respuesta de inmunidad vegetal, en donde los compuestos generados realizan funciones diversas (Torres y col., 2006). Dada su toxicidad, la acumulación de ROS tiene un efecto antimicrobiano directo contribuyendo a la muerte de los patógenos, así como un papel indirecto, al provocar la muerte de las células infectadas, impidiendo así a los patógenos el acceso al material nutritivo necesario para su crecimiento. Además, la producción de ROS, contribuye al reforzamiento de la pared celular que dificulta la progresión del patógeno en los tejidos infectados (Lamb y Dixon, 1997; Apel y Hirt, 2004; Laloi y col., 2004; Gechev y col., 2006; Triantaphylidés y Havaux, 2009; Nanda y col., 2010). Finalmente, y debido a la necesidad de limitar los efectos negativos asociados a la generación de ROS, las plantas han desarrollado mecanismos de regulación precisos que permiten controlar su producción y acumulación, y utilizar dichos compuestos como señales celulares, así como reguladores de las condiciones redox adecuadas para facilitar la activación de una respuesta de defensa eficaz (Torres y col., 2006; Breusegem Van y col., 2008; Tada y col., 2008).

Durante el estallido oxidativo, el oxígeno molecular puede convertirse en ion superóxido (O_2^-) y peróxido de hidrógeno (H_2O_2), mediante una reacción de transferencia de electrones; o en oxígeno singlete ($^1O_2$), mediante un proceso de transferencia de energía (Apel y Hirt, 2004). Los estudios transcriptómicos realizados con objeto de examinar las respuestas celulares frente a estos compuestos, han demostrado su acción como señales activando la expresión de un gran número de genes vegetales, de los que algunos son comunes y se inducen en respuesta a todos ellos, mientras que parte de la respuesta se activa de forma específica en respuesta a cada uno de ellos (O_2^-, H_2O_2 y $^1O_2$). Además, la utilización de plantas mutantes afectadas en la señalización de estos

compuestos, ha puesto de manifiesto que las rutas de señalización que regulan su acción, interaccionan entre sí de forma no conocida por el momento.

Las investigaciones dirigidas a examinar el papel de las ROS en la defensa vegetal han estado preferentemente dirigidas a estudiar la función del O_2^- y del H_2O_2. Sin embargo, resultados recientes, así como los obtenidos en este trabajo, revelan la acción directa o indirecta del oxígeno singlete ($^1O_2$) en la respuesta de la planta frente a la infección de patógenos.

1.3. PAPEL DE LAS HORMONAS EN LA RESPUESTA DE DEFENSA

Dependiendo de las estrategias utilizadas durante su infección, se pueden distinguir dos tipos de patógenos. Así, se denominan patógenos biotrofos a aquellos que se alimentan de tejidos vegetales vivos, pudiendo llegar a desarrollar una estrecha relación con su hospedador y a evolucionar a biotrofos obligados, incapaces de crecer en medios sintéticos. El segundo tipo de patógenos, denominados necrotrofos, utiliza medios menos especializados durante su infección, y establece una interacción menos estrecha con la planta. Este segundo tipo de patógenos, se desarrolla sobre tejidos vegetales muertos o senescentes y, normalmente, producen toxinas que dañan el tejido vegetal para favorecer su colonización.

Los estudios realizados con objeto de caracterizar la acción de las hormonas de defensa SA, JA y ET, han puesto de manifiesto que la ruta de señalización regulada por la acción del SA está fundamentalmente asociada con la resistencia frente a patógenos biotrofos, mientras que las rutas de transducción en las que intervienen el JA y el ET median, normalmente, la resistencia a microorganismos necrotrofos, indicando que la activación de las rutas de defensa depende, en gran parte, de la forma de vida del patógeno y de su modo de infección. (López y col. 2008)

El SA constituye una de las moléculas esenciales de la resistencia vegetal frente a patógenos, desempeñando un papel relevante en respuestas tales como la activación de la resistencia inducida (Feys y col., 2001; Loake y Grant, 2007),

el establecimiento de la HR para limitar el crecimiento de patógenos virulentos en las zonas infectadas (Clarke y col., 2001; Álvarez y col., 2000; Vlot y col., 2009), y la activación de la resistencia sistémica adquirida o SAR (del inglés, systemic acquired resistance), que protege a la planta frente a infecciones secundarias (Zimmerli y col., 2001; Vlot y col., 2009).

El aumento en los niveles endógenos de SA tras el reconocimiento de un patógeno, provoca cambios esenciales en el potencial redox de la célula que dirigen importantes procesos de señalización. Dentro de los cambios caracterizados, cabe resaltar que la reducción de la proteína NPR1, esencial en esta ruta de señalización, provoca su desagregación en el citoplasma celular, donde se encuentra formando oligómeros, a su forma monomérica activa, en la que se trasloca al núcleo para actuar como coactivador transcripcional, potenciando la unión de factores de transcripción del tipo TGA a los elementos reguladores de los promotores de los genes de respuesta a SA (Dong, 2004; Spoel y col., 2009).

Al igual que el SA, el JA regula la inducción de un importante número de genes de defensa. Estudios recientes han permitido determinar que la activación de la señalización dependiente del JA ocurre tras la interacción de esta hormona con la proteína COI1 del complejo de ubiquitinación SCF^{COI1}. Esta interacción dirige la degradación de los represores JAZ, y la liberación de factores de transcripción tales como las proteínas MYC2, ERF1 o ORA59, que actuarán a su vez modificando la expresión de los genes de defensa correspondientes (Staswick, 2008; Fonseca y col., 2009; Koo y Howe, 2009; Browse, 2009; Wu y Baldwin, 2010).

Finalmente, podemos mencionar que la ruta de señalización dependiente del ET ha sido ampliamente caracterizada (Guo y Ecker, 2003; Guzmán y Ecker, 1990; López y col., 2008; Wang y col., 2004) y que esta hormona actúa de manera coordinada (Onkokesung y col., 2010), generalmente de forma sinérgica, con el JA (Penninckx y col., 1998). La percepción del ET se establece a través de receptores, como el ETR1, localizados en la membrana del retículo endoplasmático (Kendrick y Chang, 2008), que en ausencia de la hormona

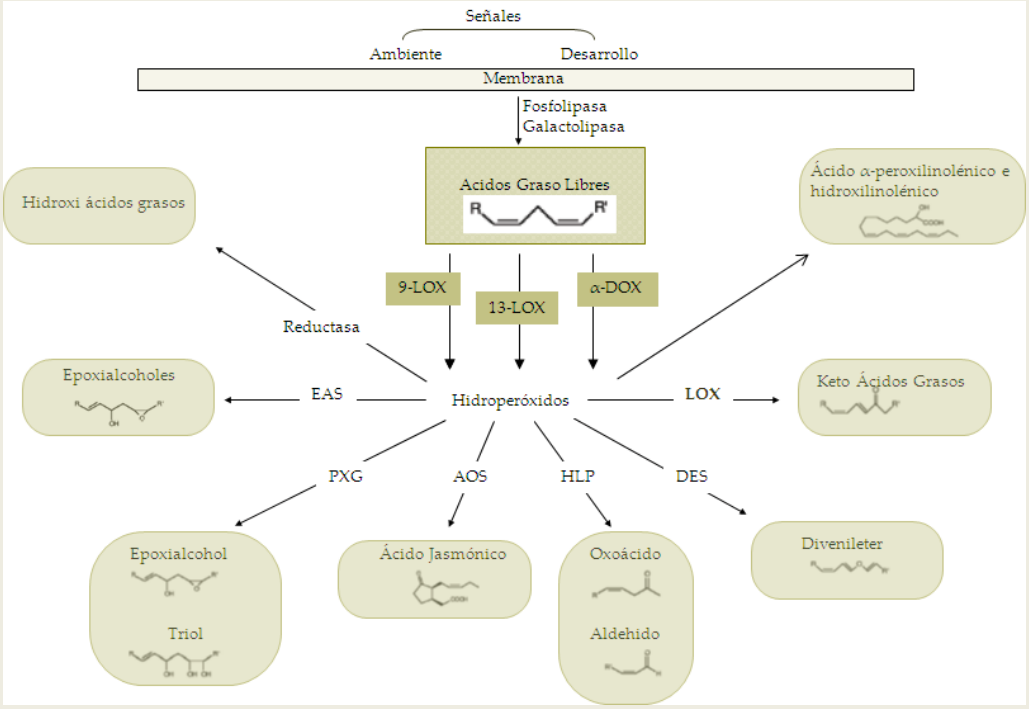
actúan como reguladores negativos de la señalización. Dicha represión, se libera al aumentar la concentración de ET en la célula, permitiendo la señalización aguas abajo a través de la activación de la proteína EIN2, que controla la mayor parte de las respuestas reguladas por esta hormona. Posteriormente, la estabilización de EIN3 conduce a la activación de un número importante de factores transcripcionales de tipo ERF, tanto activadores como represores, que serán en último término los responsables de regular la expresión de los genes dependientes de ET (Broekaert y col., 2006; Kendrick y Chang, 2008; Yoo y col., 2009).

2. OXIGENACIÓN DE ÁCIDOS GRASOS Y SÍNTESIS DE OXILIPINAS

La liberación de los ácidos grasos que forman parte de las membranas celulares, en especial de los ácidos linolénico y linoleico, constituye una de las primeras respuestas de las plantas frente a la infección de patógenos. Estos compuestos constituyen los sustratos de enzimas con actividad oxigenasa, que catalizan la incorporación de oxígeno en distintas posiciones de los ácidos grasos, iniciando rutas bioquímicas complejas a través de las que se sintetizan numerosos metabolitos de naturaleza lipídica, denominados oxilipinas, que actúan como señales celulares contribuyendo a la activación de la respuesta de defensa vegetal (Feussner y Wasternack, 2002; Shah, 2005; Howe y Jander, 2008).

Los estudios dirigidos a identificar las oxilipinas que se acumulan en la planta en respuesta a la infección de patógenos, así como de los genes que codifican las enzimas que participan en su producción, han permitido determinar la existencia de al menos tres rutas de síntesis de oxilipinas distintas, que se inician por la acción de oxigenasas de ácidos grasos con actividades 9-lipoxigenasa (9-LOX), 13-lipoxigenasa (13-LOX) y α-dioxigenasas (α-DOX). Estas enzimas catalizan la incorporación de oxígeno en las posiciones 9, 13 y 2 de los ácidos grasos sobre los que actúan, para generar los correspondientes 9,

13 y 2 hidroperóxidos que constituyen, por tanto, los productos primarios de dichas actividades enzimáticas. Los hidroperóxidos producidos se modifican posteriormente por la acción de actividades enzimáticas secundarias, tales como alenóxido sintasas (AOS), divinil éster sintasas (DES), hidroperóxido liasas (HPL), peroxigenasas (PXG), epoxi alcohol sintasas (EAS), las propias LOX y peroxigenasas/epóxido hidrolasas (Bleé, 1998; Mosblech y col., 2009). La acción conjunta de estas actividades enzimáticas da lugar a la producción de una amplia familia de derivados lipídicos oxigenados constituida por al menos 100 compuestos con distinta estructura molecular y, por tanto, con capacidad para realizar distintas acciones defensivas.



Esquema de la biosíntesis de oxilipinas en plantas

Además de a través de las actividades enzimáticas mencionadas, la incorporación de oxígeno en los ácidos grasos linolénico y linoleico puede ocurrir de forma no-enzimática, mediante oxidación química, como consecuencia de la interacción de los ácidos grasos con especies reactivas de oxígeno, tales como

los radicales hidroxilo y el oxígeno singlete. La oxidación no enzimática de ácidos grasos da lugar a la producción de un grupo específico de oxilipinas, entre las que los denominados fitoprostanos han sido los más caracterizados (Mueller and Berger, 2009).

La síntesis de oxilipinas forma parte de los programas de desarrollo de la planta, así como de la respuesta a varios estreses tanto bióticos como abióticos.

El análisis de las actividades enzimáticas involucradas en la síntesis de oxilipinas ha permitido comprobar que, en la mayoría de los casos, están codificadas por familias multigénicas cuya identificación es indispensable para analizar su funcionalidad. Los estudios realizados con objeto de identificar los genes que codifican las enzimas α-DOXs y LOXs en plantas de Arabidopsis nos han permitido identificar la presencia de dos genes *α-DOX*, denominados *α-DOX1* y *α-DOX2*, así como de seis genes lipoxigenasa de los que los designados como *LOX1* y *LOX5* codifican para enzimas con actividad 9-lipoxigenasa, mientras que los cuatro restantes (*LOX2*, *LOX3*, *LOX4* y *LOX6*) codifican enzimas con actividad 13-lipoxigenasa (Bannenberg y col., 2009). La actividad LOX en plantas de Arabidopsis está principalmente asociada a la envoltura y al estroma de los cloroplastos, mientras que, por el momento, se desconoce la localización celular de las enzimas α-DOXs. (Peltier y col. 2004)

Al igual que ocurre con las enzimas α-DOX y LOX, existen numerosos estudios dirigidos a identificar los genes que codifican cada una de las actividades enzimáticas involucradas en la modificación y síntesis de las diferentes oxilipinas que se sintetizan en la planta. Los resultados de estos estudios han permitido comprobar que estas enzimas están también codificadas por familias génicas, y que no todas las actividades enzimáticas están presentes en todas las especies vegetales. En este sentido cabe mencionar la ausencia de la actividad divinil éter sintasa (CYP74D) en el genoma de Arabidopsis (Blée y col. 2002).

Finalmente, es importante resaltar que el estudio de las rutas biosintéticas involucradas en la síntesis de oxilipinas y la cuantificación de los productos derivados, ha puesto de manifiesto que las rutas caracterizadas no son

independientes, sino que interaccionan entre sí, estableciendo una red metabólica que aumenta la flexibilidad de las plantas en su respuesta frente a distintos estímulos. Esta circunstancia, aunque beneficiosa para la adaptación de las plantas a distintas situaciones de estrés, dificulta de forma significativa la asignación de una función específica a cada una de las rutas y enzimas caracterizadas (Hamberg y col., 2003; Halitschke y col., 2004; Vellosillo y col., 2007). En este sentido cabe destacar, la interacción de las enzimas 9-LOX y α-DOX, que actúan de forma coordinada durante la síntesis de derivados lipídicos doblemente oxigenados, y que también antagonizan entre sí mediante la competición por un sustrato común y por los derivados oxigenados producidos por la acción de cada una de ellas (Hamberg y col., 2003; Vellosillo y col., 2007).

2.1. FUNCIÓN DE LAS OXILIPINAS

El reciente interés en el estudio de las oxilipinas, ha provocado un importante avance en nuestro conocimiento sobre la naturaleza de dichas moléculas, su producción y las acciones que realizan. Los resultados disponibles han puesto de manifiesto, que la expresión de una gran parte de los genes involucrados en la síntesis de oxilipinas se induce en la planta en respuesta a la infección por distintos patógenos, dando lugar a la acumulación de grupos específicos de oxilipinas que contribuyen a la defensa de la planta frente a distintos tipos de patógenos (Göbel y col., 2001; Ponce de León y col., 2002; Andersson y col., 2006).

De forma generalizada, la caracterización funcional de oxilipinas ha permitido distinguir la capacidad de determinados derivados para ejercer tres tipos de actividades defensivas de la planta: una actividad señalizadora que conduce a la inducción de genes específicos de la planta, una actividad reguladora del proceso de muerte celular que acompaña a la inducción de la respuesta de defensa vegetal y, finalmente, una acción directa como moléculas con actividad antimicrobiana (Vollenweider y col., 2000; Stintzi y col., 2001;

Montillet y col., 2005; Prost y col., 2005; Andersson y col., 2006; Kishimoto y col., 2008).

Dentro de esta familia de metabolitos, los jasmonatos han sido extensamente caracterizados y su participación en distintas respuestas, tales como la defensa frente a necrotrofos, la respuesta a herida mecánica, la protección frente a insectos y la maduración del polen, ha sido ampliamente demostrada (Staswick, 2008; Fonseca y col., 2009; Koo y Howe, 2009; Wu y Baldwin, 2010; Browse, 2009). Además, los estudios realizados han permitido asignar funciones relevantes a otras oxilipinas generadas, tanto a través de la ruta iniciada por la acción de las enzimas 13-LOX, como a través del resto de las rutas involucradas en la síntesis de oxilipinas. Así, una parte de los compuestos sintetizados a través de la ruta de las 13-LOXs, tales como el 12-OxoPDA, precursor del JA, y otros derivados C6 producidos por la acción de la 13-HPL, actúan como reguladores de la expresión de genes de defensa (Farmer y col., 2003), mientras que los correspondientes KOTs y KODs contribuyen tanto a la inducción de genes de defensa, como a la activación de procesos de muerte celular. Por otro lado, los derivados 13-HPOT y 13-HOT actúan también como inductores de la expresión génica y como compuestos antimicrobianos inhibiendo el crecimiento de patógenos vegetales (Weichert y col., 1999; Vollenweider y col., 2000; Graner y col., 2003; Prost y col., 2005). Otros ejemplos de oxilipinas con actividad antimicrobiana son el *cis*-3-hexenol y el *trans*-2-hexenal, derivados igualmente de la ruta iniciada por la acción de las 13-LOX (Croft y col., 2003; Prost y col., 2005).

Además, existen datos recientes que demuestran la participación de las rutas iniciadas por la acción de las α-DOXs y 9-LOXs, así como de los productos sintetizados a través de dichas rutas, en la defensa vegetal. Así, el estudio de las enzimas α-DOX reveló la participación de la proteína α-DOX1 en la defensa de la planta frente a la infección de patógenos biotrofos, y su función en el control del daño celular asociado con la generación del estrés oxidativo, que acompaña a la inducción de la defensa vegetal frente a este tipo de patógenos (Sanz y col., 1999; Ponce de León y col., 2002; Hamberg y col., 2003). Igualmente, el estudio

de esta actividad enzimática ha puesto de manifiesto su participación en la respuesta de las plantas frente al ataque de insectos (Steppuhn y col., 2010). Del mismo modo, el papel de la ruta 9-LOX en la defensa de tabaco y Arabidopsis se ha puesto de manifiesto mediante la utilización de plantas transgénicas, alteradas en los niveles de expresión de los genes correspondientes, y de plantas mutantes, carentes de dichas actividades enzimáticas (Rancé y col., 1998; Seo y col., 2001; Mene-Saffrane y col., 2003; Andersson y col., 2006; Vellosillo y col., 2007; Hwang y Hwang, 2010; López y col., 2011). Al igual que se describe anteriormente, los compuestos derivados de estas rutas biosintéticas actúan como reguladores de la expresión génica y como compuestos antimicrobianos (Bleé, 1998; Göbel y col., 2002; Weber y col., 1999; Prost y col., 2005; Vellosillo y col., 2007; Hwang y Hwang, 2010; López y col., 2011). Así mismo, estudios adicionales han demostrado la acción defensiva de estos derivados al actuar como reguladores de la formación de los síntomas y de los cambios hormonales producidos por los patógenos durante la infección (Hamberg y col., 2003, Vicente et al., 2012).

Además de su papel en la respuesta de defensa y, al igual que en el caso de las oxilipinas derivadas de la actividad 13-LOX, la caracterización de las enzimas α-DOX y 9-LOX ha puesto de manifiesto su participación en procesos de desarrollo. En concreto el papel de la enzima α-DOX2 en el desarrollo de las plantas de tomate se concluye de los resultados que demuestran que la falta de esta enzima provoca graves alteraciones fenotípicas, que afectan a todos los estadios del desarrollo de la planta. Una situación semejante ocurre en las plantas de Arabidopsis, en donde la expresión del gen *α-DOX2* no se activa en respuesta a patógenos, sino que varía de acuerdo al estado de desarrollo de la planta, aunque a diferencia de lo que ocurre en plantas de tomate, la falta de actividad α-DOX2 en Arabidopsis no está acompañada de alteraciones fenotípicas visibles. Finalmente cabe mencionar el papel de las enzimas 9-LOX en el desarrollo radicular en donde su actividad contribuye a regular el proceso de emergencia de las raíces laterales (Vellosillo y col., 2007).

2.2. OXILIPINAS Y ESPECIES REACTIVAS DE OXÍGENO

Además de las rutas de sintesis de oxilipinas descritas anteriormente, la generación de oxilipinas se produce, tambien, de forma no enzimatica como resultado de la interacción de los ácidos grasos, linolénico y linoleico, con especies reactivas de oxígeno, tales como los radicales hidroxilos y el oxígeno singlete.

La oxigenación no enzimatica de acidos grasos da lugar a la producción de oxilipinas específicas entre las que los fitoprostanos han sido los más caracterizados (Loeffler y col., 2005). A diferencia de estos compuestos, las oxilipinas producidas por la acción del oxigeno singlete han recibido menor atención, aunque su participación en la respuesta de la planta frente a distintos tipos de estrés se ha puesto de manifiesto recientemente. Asi, estudios publicados en los últimos años han demostrado la acumulación de derivados hidroxilados, generados de forma específica por la acción del oxigeno singlete, tales cono el 10-HOD, 10-HOT, 12-HOT y 15-HOT, durante la adaptación del mutante *flu* a los cambios de luz. En estos estudios se ha demostrado, que el nivel de los derivados hidroxilados generados, determina su acción como compuestos toxicos que contribuyen a la formación de lesiones necróticas en las células afectadas, o como señales reguladoras de los procesos celulares que facilitan la adaptación de la plantas a los cambios de luz (Przybyla y col., 2008).

Los resultados que demuestran la acumulación de derivados hidroxilados, generados por la acción del oxígeno singlete, en hojas de Arabidopsis infectadas con *Pseudomonas syringae* pv *tomato,* sugieren la participación de estos compuestos en la defensa de la planta (Mueller y col., 2008). En apoyo de esta idea, resultados de nuestro laboratorio han puesto de manifiesto que la aplicación de $^1O_2$-hidroxi ácidos, provoca la formación de depositos de calosa en hojas (12 HOT) y raíces (10-HOD, y 12 KOT) de Arabidopsis (Vellosillo et al., 2011). Igualmente resultados no publicados de nuestro grupo (Kulasekaran y Castresana, en preparación) permiten comprobar que la aplicación de 10-HOT, en plantas silvestres de Arabidopsis, provoca una importante reprogramación

transcripcional que afecta a la expresión de numerosos genes de la planta, entre los que un alto porcentaje de ellos corresponde a genes de defensa vegetal cuya expresion se induce durante el tratamiento examinado.

Los resultados descritos a lo largo de esta introducción ponen de manifiesto la participación de las oxilipinas como parte de los mecanismos de defensa de la planta frente a la infección de microorganismos patógenos. Sin embargo, y a pesar de los recientes avances en el estudio de estos compuestos, nuestro conocimiento acerca de las acciones que realizan, así como de los mecanismos a través de los que ejercen dichas acciones, es todavía limitado. Por ello, y en base a nuestro interés en el estudio de esta familia de metabolitos, en el presente trabajo nos propusimos profundizar en la caracterización de la ruta de síntesis de oxilipinas iniciada por la acción de los enzimas 9-LOX, con objeto de determinar su función y, por tanto, la de los compuestos derivados correspondientes. Para desarrollar este objetivo hemos desarrollado una estrategia genética en la que hemos procedido a aislar y caracterizar plantas mutantes carentes de dicha actividad enzimática y, por tanto, de las oxilipinas procedentes de su acción y, por otro, plantas mutantes afectadas en los procesos de señalización que regulan la respuesta de las plantas a la aplicación de las oxilipinas generadas a traves de esta ruta biosintética. En nuestro estudio hemos examinado la respuesta de ambos tipos de mutantes frente a patógenos y determinado la contribución de los genes corerspondientes en los mecanismos de defensa de la planta.

OBJETIVOS

OBJETIVOS

El presente trabajo ha sido planteado con objeto de profundizar en la caracterización funcional de la ruta de síntesis de oxilipinas, iniciada por la acción de las enzimas 9-LOX, durante la respuesta de la planta frente a la infección de bacterias hemibiotrofas del género *Pseudomonas*. Para ello hemos desarrollado una estrategia genética dirigida a obtener y caracterizar dos tipos de plantas mutantes: i) plantas carentes de actividad 9-LOX y ii) plantas alteradas en los procesos de señalización que se activan en respuesta a oxilipinas derivadas de la acción de las 9-LOX, y en particular en la respuesta de la planta a la aplicación de ácido 9-hidroxytrienoico (9-HOT). El crecimiento de plántulas de *Arabidopsis* en presencia de 9-HOT induce un fenotipo denominado ¨waving radicular¨ que ha sido utilizado para la selección de plantas mutantes insensibles a 9-HOT, y por tanto incapaces de inducir waving radicular en respuesta a este compuesto. Dos de dichos mutantes, denominados *noxy6* y *noxy22* (del inglés *non responding to oxylipins*), han sido seleccionados para su estudio en este trabajo. La respuesta de los mutantes seleccionados frente a la infección de bacterias virulentas y avirulentas ha sido examinada en detalle, en comparación con la correspondiente a la de las plantas silvestres. Los resulatdos de este trabajo han aportado nueva información acerca del papel de las oxilipinas examinadas en la respuesta de la planta frente a la infección de bacterias hemibiotrofas, asi como su interacción con la ruta de señalización regulada a través de la acción del etileno.

MATERIALES Y MÉTODOS

MATERIALES Y MÉTODOS

1. ESTIRPES BACTERIANAS

1.1. CEPAS BACTERIANAS

1.1.1. Bacterias no fitopatógenas

Escherichia coli. Se utilizó la cepa DH5α (Hanahan y col., 1983) para la propagación y purificación de plásmidos, que se cultivó a 37°C en medio LB con los antibióticos necesarios para cada caso.

Agrobacterium tumefaciens. La cepa C58C1 de *Agrobacterium tumefaciens* conteniendo el plásmido pGV2260, se empleó para la transformación de las plantas con las construcciones de interés (Deblaere y col., 1985). Las bacterias se cultivaron a 28°C en medio LB, en presencia de rifampicina (100 mg/L) utilizado como antibiótico de selección.

1.1.2. Bacterias fitopatógenas

Las bacterias fitopatógenas utilizadas fueron *Pseudomonas syringae pv. tomato* DC3000 *avr*Rpm1 (Debener y col., 1991; Innes y col., 1993) y *Pseudomonas syringae pv. tomato* DC3000 (Whalen y col., 1991) ambas suministradas por el Profesor J. Dangl (Universidad de Carolina del Norte).

1.2. MEDIOS DE CULTIVO BACTERIANOS

Medio Luria-Bertani (LB): 10 gramos de Bact-triptona (1%), 5 gramos de extracto de levadura (0,5%), 10 gramos de NaCl (1%), completar con agua milliQ, ajustar el pH a 7,2 con NaOH. Para el cultivo en medio sólido añadir 15 gramos de agar.

Medio de cultivo Kings-B (KB): 20 gramos de Proteosa Peptona 3, 17,2 mililitros de Glicerol 87%, 15 gramos de Agar (únicamente para cultivo sólido). Tras autoclavar añadir 6,5 mililitros de K_2HPO_4 (6,5mM), 6 mililitros de SO_4Mg+7H_2O (6mM).

Medio SOB: 2% de tritona, 0.5% de extracto de levadura, 0.05% de NaCl, 250 mM de KCl.

Medio SOC: 97% de medio SOB, 2% de glucosa 1M, 1% de Cl_2Mg 1M.

1.3. MANTENIMIENTO DE LAS CEPAS BACTERIANAS

E. coli se cultivó a 37°C en medio LB, añadiendo 15 g/L de agar para cultivos en medio sólido.

Los cultivos de cepas de *Pseudomonas* se crecieron en medio King´s B (King y col., 1954), añadiendo 15 g/L de agar para cultivos en medio sólido, en presencia de los antibióticos correspondientes, a una temperatura de 28°C.

Los cultivos de cepas originales y los transformantes de *A. tumefaciens* se realizaron a 28°C en medio LB.

Las cepas originales y los transformantes de *E. coli* y *A. tumefaciens*, así como las bacterias fitopatógenas utilizadas se mantuvieron, durante periodos cortos de tiempo, a 4°C en placa de medio sólido que contenían los antibióticos apropiados.

1.4. MEDIO DE CONSERVACIÓN BACTERIANA

Para el mantenimiento indefinido se guardaron las cepas en su propio medio de cultivo glicerinado a -80ºC con una concentración de glicerol estéril del 15%.

1.5. MEDIOS DE SELECCIÓN BACTERIANA

Cualquier cepa debe ser sometida a selección con el fin de mantener un vector plasmídico. El procedimiento de selección más generalizado en organismos procariotas es la resistencia a antibióticos que muestran las cepas de interés gracias a productos génicos presentes en los plásmidos transformados.

Dado que los antibióticos, por lo general, no soportan temperaturas superiores a 60º C han de ser añadidos, desde una solución madre esterilizada por filtración con filtros de 0,22µm de diámetro de poro, sobre los medios de cultivos ya autoclavados. Los medios de selección utilizados en este trabajo fueron los apuntados en la tabla 1.

1.6. MANIPULACIÓN GENÉTICA DE ESTIRPES BACTERIANAS

1.6.1. Obtención de células competentes de *E.coli*.

A partir de un stock mantenido a -80ºC de células de *E.coli* se sembraron, haciendo estrías, una placa de LB y se dejaron crecer durante 24 horas a 37ºC.

A partir de una colonia del cultivo se inocularon 200ml de medio SOB y se dejaron crecer a 22ºC en agitación hasta que el cultivo alcanzó una densidad óptica de 0.6 leído en un espectrofotómetro a 600nm. Cuando el cultivo se

encontró en fase exponencial se pasó a hielo durante 10 minutos, a continuación se centrifugó a 4ºC a una velocidad de 3000 r.p.m. durante 10 minutos, posteriormente se descartó el sobrenadante y se resuspendió el precipitado en 200 mL de TB frío. Se añadió DMSO hasta alcanzar una concentración del 7% agitando gentilmente. Finalmente, tras incubar en hielo durante 10 minutos, se repartieron las células competentes en alícuotas de 200 μL, se congelaron inmediatamente en nitrógeno líquido y se guardaron a -80ºC.

1.6.2. Obtención de células competentes de *A.tumefaciens*.

A partir de un stock mantenido a -80ºC de células de *A.tumefaciens* se sembró, haciendo estrías, una placa de LB y se dejó crecer durante 48 horas a 28ºC.

Partiendo de una colonia aislada se preparó un preinóculo 5 mL de LB, manteniéndolo durante toda la noche a 28ºC y agitación débil. Después se introdujo el preinóculo en 200 mL de LB y se dejó crecer a 28ºC en agitación durante 5-6 horas. A continuación se centrifugó el cultivo a 5000 r.p.m. durante 10 minutos a 4ºC, se descartó el sobrenadante y se resuspendió el precipitado en 100 mL de Tris-HCl 100 mM pH8.0 estéril. Posteriormente se volvió a centrifugar en las mismas condiciones, se descartó el sobrenadante y se resuspendió el precipitado en 2 mL de LB. Por último, se repartió el cultivo en alícuotas de 200 µL, se congelaron en nitrógeno líquido y se almacenaron a -80ºC.

1.6.3. Transformación de *E.coli*.

La transformación de células competentes de *E.coli* se realizó según se describe en Sambrook y col., (1989).

En primer lugar se descongelaron las células en hielo durante aproximadamente 10 minutos, se añadieron las ligaciones (inserto de ADN y el plásmido de interés) y se mantuvieron en hielo durante 30 minutos. A continuación se sometió la mezcla a un choque térmico poniéndola a 42ºC

durante 90 segundos en un termobloque, posteriormente se enfriaron las células en hielo durante 2 minutos. Estos cambios bruscos de temperatura consiguen desestabilizar las membranas y facilitar la entrada de las ligaciones a la célula. Después, se añadieron 0,8 ml de medio SOC y se incubaron durante una hora a 37ºC. Tras esta primera incubación se sembraron en medio LB sólido suplementado con el agente selectivo, determinado por el plásmido a transferir. Finalmente se incubaron las células a 37ºC durante una noche y se eligieron las colonias de bacterias transformadas.

1.6.4. Transformación de *A.tumefaciens*.

Se descongeló una alícuota (200µL) de células competentes de *A.tumefaciens* en hielo, una vez descongelada se añadieron entre 0,1µg y 1 µg del ADN plasmídico de interés y se mantuvo en hielo 5 minutos más. A continuación se sometió las células competentes a un choque térmico introduciendo la mezcla en nitrógeno líquido durante 5 minutos y pasándola rápidamente a 37ºC durante 5 minutos más. Posteriormente se añadió 1 ml de LB y se incubó a 28ºC durante 1 hora en agitación. Por último se plaquearon alícuotas de 300 µL en placas de LB suplementadas con el antibiótico de selección, se dejaron creciendo a 28ºC durante 48 horas y se seleccionaron las bacterias transformantes.

1.7. CÉLULAS DE INSECTO

Para la expresión de proteínas recombinantes se emplearon células de insecto “High Five” (BTI-TN-5B1-4; Invitrogen), una línea celular derivada originalmente de *Trichoplusia ni.* Las células fueron cultivadas en condiciones estériles a 28ºC, en medio Tc100 suplementado con 5% de suero bovino fetal (inactivado por calor) y antibióticos. El antibiótico empleado fue: gentamicina (50 µg/mL). Las células fueron crecidas en placas Petri de plástico (Nunc, Roskilde, Dinamarca; no. 168381; diámetro 150 mm) con 18 mL de medio. Las células fueron subcultivadas cuando llegaron a 90-100% de confluencia. Las células

fueron infectadas con baculovirus a una confluencia de aproximadamente el 80%.

2. MATERIAL VEGETAL

En este trabajo se utilizó *Arabidopsis thaliana* ecotipo Col-0.

Se emplearon plantas transgénicas portadoras de las construcciones: *LOX1::GUS, LOX2::GUS, LOX3::GUS, LOX4::GUS, LOX5::GUS, LOX6::GUS*.

Otras plantas transgénicas utilizadas en este trabajo fueron las portadoras de la construcción *35S:EIN3:GFP* y EBS::GUS suministradas por el Prof. J.R.Ecker y Prof. J. Alonso, respectivamente.

Se utilizaron mutantes de inserción de ADN-T SALK_059431 (*lox1-1*), *locus* At1g55020 y SALK_044826 (*lox5-1*), *locus* At3g22400, pertenecientes a la colección de líneas de ADN-T del Salk Institute (Alonso y col., 2003) que fueron proporcionados por el NASC (Nottingham Arabidopsis Stock Center). También se utilizó el mutante *ein2-5* cedido por el Dr. R. Solano.

Además se empleó para la búsqueda de mutantes alterados en la respuesta a 9-HOT, una población M2 de semillas mutadas por metanosulfonato de etilo, adquiridas en la empresa Lehle Seeds.

2.1. MEDIOS DE CULTIVO VEGETALES

2.1.1. Cultivo in vitro

Se empleó medio Murashige & Skoog (4,4g/L) con 1% de sacarosa y 0,7% de agar para cultivo en posición horizontal y 1,5% para los experimentos en posición vertical, dicho medio se ajustó a pH 5,7 con NaOH.

2.1.2. Cultivo en tierra

Para el cultivo de plantas en tierra se empleó sustrato con 3 partes de turba sin fertilizar y 1 parte de vermiculita.

2.2. CONDICIONES DE CULTIVO DEL MATERIAL VEGETAL

2.2.1. Crecimiento en tierra

Las semillas de *A.thaliana* se sembraron en bandejas de 54 alveolos de 80 dm^3 de volumen cada uno y se estratificaron durante 4 días a 4°C tras la imbibición.

Las semillas estratificadas se pusieron a crecer en cámaras de condiciones ambientales controladas a 22° de temperatura, 70% de humedad relativa y 200 µE M2 sec1 de iluminación fluorescente, bajo un fotoperiodo de 14 horas de luz y 10 horas de oscuridad.

Los distintos tratamientos de plantas adultas se realizaron en plantas de 4 semanas de edad

2.2.2. Crecimiento *in vitro*

Para los experimentos de cultivo de plantas *in vitro*, la superficie de las semillas de *Arabidopsis thaliana* se esterilizó usando hipoclorito sódico al 75% (v/v) en agua más 0,001% de Tween 20 durante 7 minutos, a continuación se lavaron las semillas 4 veces con agua estéril. Se estratificaron durante 3 días a 4ºC. Los experimentos se realizaron en placas de 12x12 cm con 1 cm de medio de cultivo. Las placas se cerraron con cinta quirúrgica porosa Micropore y se pusieron a crecer a 0º o 90º de inclinación con respecto a la vertical en cámaras climáticas de ambiente controlado en condiciones de 22° de temperatura, 50% de humedad relativa y 200 µE M2 sec1 de iluminación fluorescente, bajo un fotoperiodo de 14 horas de luz y 10 horas de oscuridad.

Para el crecimiento en medio líquido se esterilizaron las superficies de las semillas como se ha descrito anteriormente y se crecieron un máximo de 20 semillas en placas multipocillos de 12 alveolos (Nunc) con un volumen de 3mL de medio MS por alveolo. Los distintos tratamientos se realizaron en plántulas de 7 días de edad.

2.2.3. Plásmidos

PGEMT-easy (Promega): utilizado para la clonación de fragmentos de ADN obtenidos por PCR. El vector comercial posee timinas desapareadas en sus extremos 5' que hibridan con las adeninas que incorpora el enzima Taq polimerasa en las reacciones de amplificación, facilitando así la clonación de los productos de PCR. Contiene un gen de resistencia a ampicilina y el gen lacZ que permite la selección azul-blanca de las cepas transformantes.

pBI101.2: Plásmidos derivados de pBin19 (Bevan, 1984) que contienen la secuencia del gen de la *β-glucuronidasa* (*GUS*). Al no presentar promotor se emplean para el análisis, a través de la expresión de *GUS*, de secuencias promotoras de genes de interés (Jefferson y col., 1987).

pFascBac: vector de expresión en el que la expresión del gen de interés está controlada por un promotor específico de baculovirus, el promotor del gen de la polihedrina del virus de la polihedrosis nuclear de *Autographa califórnica* (AcNPV). Como marcadores contiene el gen de resistencia a ampicilina, el gen de resistencia a gentamicina y el gen de resistencia a kanamicina. Este vector contiene el promotor AcNPV, el gen de resistencia a gentamicina, un sitio de clonaje múltiple, en el que se inserta el gen de interés, y la señal de poli(A) de SV40 (mini-TN7), entre los brazos izquierdo y derecho del elemento transponible Tn7.

3. TÉCNICAS DE BIOLOGÍA MOLECULAR

3.1. PREPARACIÓN Y ANÁLISIS DE ÁCIDOS NUCLEICOS

3.1.1. Preparación de ADN genómico de plantas

La preparación de ADN genómico de plantas se realizó siguiendo el método descrito por Dellaporta y col. (1983). El ADN obtenido se cuantificó mediante espectrofotometría midiendo la absorción a una λ=260 nm (DO=1 equivale a 50 mg/mL) y se analizó por electroforesis y tinción con BrEt.

Para la obtención de ADN a partir de un alto número de muestras, se utilizó el kit "BioSprint DNA Plant" (Qiagen), basado en la tecnología de partículas magnéticas, y el robot BioSprint 96, siguiendo las instrucciones del fabricante.

3.1.2. Aislamiento y purificación de plásmidos

Para la purificación de ADN plasmídico a pequeña escala (5 mL de cultivo bacteriano) se utilizó el método de lisis alcalina con el kit "QIAprep Spin Miniprep Kit" (Qiagen). Las preparaciones a gran escala (100 mL) se realizaron por cromatografía, utilizando las columnas suministradas en el kit "Plasmid Midi Kit" (Qiagen).

3.1.3. Elución de fragmentos de ADN

El aislamiento de los fragmentos de ADN de interés se realizó mediante migración en geles de agarosa y posterior elución empleando las columnas del kit "QIAquick Gel Extraction" de Qiagen.

3.1.4. Amplificación de ADN mediante la reacción en cadena de la polimerasa (PCR).

Las amplificaciones de ADN mediante la reacción de polimerización en cadena o PCR (Saiki y col., 1985) se realizaron en un termociclador GeneAmp PCR System 9700 (Applied Biosystems).

3.1.5. Síntesis de oligonucleótidos

Los oligonucleótidos empleados en las reacciones de PCR y en las secuenciaciones se obtuvieron de las casas comerciales Roche Applied Science, Invitrogen y Sigma Aldrich.

3.1.6. Tratamiento enzimático del ADN

La manipulación enzimática del ADN, con enzimas de restricción u otras enzimas modificadoras, se hizo atendiendo a las especificaciones del proveedor y de acuerdo a los protocolos descritos por Sambrook y col. (1989). Posteriormente, las enzimas se retiraron de la reacción mediante extracción con fenol-cloroformo-isoamílico (25:24:1).

3.1.7. Ligación de fragmentos de ADN

Las reacciones de ligación de fragmentos de ADN obtenidos por digestión con enzimas de restricción se llevaron a cabo utilizando la enzima ADN ligasa del bacteriófago T4 (Roche) y siguiendo las instrucciones del fabricante.

3.1.8. Secuenciación de ADN

La secuenciación del ADN se llevó a cabo en el servicio de secuenciación de la empresa Secugen. En todos los casos se suministró al servicio de secuenciación ADN plasmídico, o fragmentos obtenidos mediante PCR, purificados con el kit "QIAquick PCR Purification" (Qiagen).

3.1.9. Análisis de secuencias de ADN

Las secuencias se analizaron mediante los programas Chromas y 4Peaks.

3.1.10. Análisis transcriptómicos

Se amplificó y se marcó el ARN total (1 µg) usando MessageAmp™ II aRNA kit (Ambion) y 5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate (aa-dUTP, Ambion), según las instrucciones del fabricante. Para cada muestra, 3,5 µg del ARN marcado se resuspendió en 0,1 M de Na_2CO_3 (pH 9,0) y se conjugó bien con Cy3 o bien con Hyper 5 Mono NHS Ester (Cy™Dye Post-labelling Reactive Dye Pack, Amersham). Las muestras fueron purificadas con MegaclearTM (Ambion) siguiendo las instrucciones del fabricante. La incorporación de Cy3 y Hyper 5 se midió mediante un espectrofotómetro Nanodrop. La preparación y la hibridación de las sondas se llevó a cabo como se describe en el manual: Two-Color Microarray Based Gene Expression Analysis Ver. 5.7, Agilent Technologies. Para cada hibridación, 825 ng de las sondas de Cy3 y Hyper 5 se mezclaron brevemente y se añadió a 11 mL del agente bloqueante 10x, 2,2µL del tampón de fragmentación 25x y agua libre de nucleasa en 55 mL de reacción, se incubó a 60ºC durante 30 minutos para romper el ARN y se paró con tampón de hibridación 2x. Las muestras se pusieron en hielo y rápidamente se cargaron en la micromatriz, se hibridaron a 65ºC durante 17 horas, transcurrido ese tiempo se lavaron una vez con tampón de lavado GE 1 a temperatura ambiente (1 minuto) y una vez con tampón de lavado GE 2 a 37ºC (1 min). La micromatriz se secó mediante centrifugación a 2000 rpm durante 2 minutos. Las imágenes de los canales Cy3 y Hyper5 se equilibraron, se capturaron con GenePix 4000B (Axon) y los puntos se cuantificaron usando el programa GenPix (Axon). El fondo se corrigió y la normalización de los datos de expresión se llevó a cabo mediante el método LIMMA (Smyth 2004; Smyth & Speed 2003). El método LIMMA es parte del proyecto Bioconductor y lenguaje R (Ihaka & Gentleman 1996). Para la corrección del fondo se usó el método "normexp" en LIMMA. El logaritmo de los ratios resultantes fue normalizado para

cada micromatriz mediante print-tip (Smyth & Speed 2003). Para obtener distribuciones similares entre las micromatrices y para conseguir valores consistentes en las micromatrices, los valores de los ratios de los logaritmos se escalaron usando el estimador de escala the median-absolute-value (Smyth & Speed 2003). El método de modelos lineales se usó para determinar los genes expresados diferencialmente. Se probaron los cambios de expresión para cada sonda en cada uno de los replicados mediante Bayes moderated t-satatistic. Para el control de FDR (false discovery rate) los *p-values* fueron corregidos mediante el uso del método de Benjamini and Hochberg (Benjamini & Hochberg 1995). Se comprobó que los FDR esperados fueran menores al 5%. Los análisis estadísticos y la representación gráfica de los datos se llevaron a cabo mediante la herramienta interactiva FIESTA (http://bioinfogp.cnb.csic.es/tools/FIESTA).

3.2. TÉCNICAS DE ELECTROFORESIS

La separación de fragmentos de ADN se realizó mediante electroforesis en geles horizontales de agarosa de concentración variable, dependiendo del tamaño de los fragmentos analizados. Se utilizó el tampón TBE (Tris-borato 45 mM; EDTA 1 mM, pH8) al 0,5%. Los fragmentos de ADN se visualizaron en un transiluminador de luz ultravioleta por tinción con BrEt 0,5 mg/L (Sambrook y col., 1989).

La resolución del ARN se llevó a cabo en geles de agarosa al 1,5% con formaldehido 6%, según se describe en Sambrook y col. (1989), y se visualizaron en un transiluminador de luz ultravioleta mediante tinción de las muestras con BrEt.

3.3. PREPARACIÓN, ANÁLISIS Y TRANSFERENCIA DE ARN

La preparación del ARN total de las plantas se obtuvo mediante la precipitación con ácido acético 1M y etanol 100%, según el protocolo descrito por Logemann y col. (1987), y se cuantificó mediante espectrofotometría a una λ=260 nm (DO=1 equivale a 40 mg/mL).

La resolución del ARN se llevó a cabo en geles de agarosa al 1,5% con formaldehido 6%, según se describe en Sambrook y col. (1989), y se visualizaron en un transiluminador de luz ultravioleta mediante tinción de las muestras con bromuro de etidio.

El ARN se transfirió por capilaridad a membranas de nylon Hybond-N (Amersham), siguiendo las instrucciones de la firma comercial. Los ácidos nucleicos se fijaron posteriormente a la membrana por exposición a la luz UV (70 mJ/cm^2).

3.4. MARCAJE RADIACTIVO E HIBRIDACIÓN DE ÁCIDOS NUCLEICOS

Las sondas de ARN radioactivo utilizadas en los ensayos tipo Northern, se sintetizaron mediante transcripción *in vitro*, en presencia de α-[32P]dCTP, utilizando el kit de transcripción de ARN (Roche). Los ADN molde se encontraban clonados en plásmidos que, previamente al marcaje, se digirieron con las enzimas de restricción adecuadas en cada caso. Se prepararon sondas radioactivas a partir de ADNc correspondientes a los genes: *PR1* (At2g14610) y *PR2* (At3g57260), *LOX1* (At1g55020), *LOX5* (At3g22400), *ABC* (At1g15520), *FOX* (At1g26390) y *POX* (At5g22140),

Las membranas con el ARN transferido se prehibridaron durante al menos 2 horas en una solución de 5% SSC, 50% formamida, 0,25 g de leche en polvo, 0,5% SDS y 20 μg/mL de ADN de esperma de salmón desnaturalizado. Las pre hibridaciones e hibridaciones se realizaron a 68°C. Tras la adición de las sondas radioactivas, los filtros se hibridaron durante un periodo de 8 a 15 horas, y se lavaron, posteriormente, por periodos de tiempo de 25 minutos en soluciones de 5% SSC, 0,1% SDS y 2% SSC, 0,1% SDS y, por último, durante 10-15 minutos con 0,1% SSC, 0,1% SDS. Todos los lavados se efectuaron a 68°C. Los filtros se dejaron secar y se expusieron a películas de autoradiografía Hyperfilm de la casa Amersham. En algunos casos, los filtros se revelaron mediante el sistema Molecular Dynamics versión 4.1STORM860 PhosphorScreen.

3.5. REACCIÓN DE RETRO-TRANSCRIPCIÓN DE ARN (RT-PCR)

Los análisis de expresión génica se realizaron, en casos específicos, mediante RT-PCR semicuantitativa a partir del ARN total de la planta. Esta reacción se llevó a cabo en un solo paso utilizando el kit "Titan One Tube RT-PCR system" (Roche Applied Science), siguiendo las instrucciones del fabricante. El ARN total de la planta fue tratado con DNase TURBO DNA-free (Ambion) para eliminar posibles contaminaciones de ADN. Para cada reacción se usó una cantidad de ARN de 100 ng. Como control interno de carga se usó el gen At1g43170 que codifica la proteína ribosomal 60S L3 (RPL3A). Los diferentes cebadores usados en esta reacción se muestran en la Tabla 2.

3.6. EXTRACCIÓN DE PROTEÍNAS Y WESTERN-BLOT

Se prepararon extractos proteicos homogenizando el tejido en un buffer de extracción que contiene 50 mM TrisHCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM PMSF y 1 unidad del “complete protease inhibitor cocktail (Roche, http://www.roche.com). Se obtuvieron los sobrenadantes mediante centrifugación a 13000 g durante 10 min, se separaron las proteínas mediante electroforesis al 10% de SDS y se realizó la transferencia a membranas de nitrocelulosa mediante electroblotting. Finalmente la incubación de las membranas con los anticuerpos correspondientes se realizó según lo descrito en Sanz y col. (1998).

3.7. SISTEMA DE EXPRESIÓN DE PROTEÍNAS EN CÉLULAS DE INSECTO CON BACULOVIRUS

3.7.1. Expresión de proteínas vegetales en células de insecto

El estudio de la actividad enzimática de las proteínas LOXs se realizó mediante expresión del correspondiente ADNc (los fragmentos y las secuencias de los cebadores utilizados están recogidos en la Tabla 3) en células de insecto infectadas con baculovirus recombinantes, siguiendo el manual de instrucciones

del Sistema de Expresión en Baculovirus Bac-to-Bac de Invitrogen (Luckow y col., 1993; Barnett y col., 1994).

Para ello el ADNc se clonó en el vector pFastBac, a continuación del promotor del gen de la polihedrina del virus de la polihedrosis nuclear *Autographa californica* (AcNPV). El plásmido recombinante obtenido se transfirió a la cepa de *E.coli* DH10BAC. Esta cepa contiene el bácmido bMON14272, con el sitio de unión del transposón bacteriano Tn7 (mini-att-Tn7), y un plásmido facilitador que permite la inserción del elemento transponible mini-Tn7, desde el plásmido donante pFastBac al sitio de unión mini-att-Tn7 del bácmido receptor. Las colonias DH10BAC que contenían los bácmidos recombinantes se identificaron mediante la interrupción de la coloración azul característica de las colonias que contenían los bácmidos originales. A partir de las colonias de *E.coli* DH10BAC seleccionadas, se realizó una minipreparación de ADN de alto peso molecular, que se utilizó para trasfectar las células de insectos (HighFive). Una vez que se había realizado la transfección de las células se recogió el sobrenadante (2 mL) que contenían los stocks de virus recombinantes, se clarificó mediante centrifugación y los distintos stocks se guardaron protegidos de la luz a -80ºC. Posteriormente, para realizar la amblificación de cada uno de los virus de los stock, se efectuó una infección de células de insecto con dichos virus a una multiplicidad de infección entre 0,001 y 0,1 ufp/células. Cultivos de células HighFive se infectaron con las correspondientes partículas de virus recombinantes a una multiplicidad de infección aproximada de 5 ufp/célula para examinar la expresión y actividad enzimática de las proteínas descritas.

3.8. MEDIDA DE LA ACTIVIDAD LOXS EN CÉLULAS DE INSECTO

Las medidas de las actividades LOXs se realizaron a partir de 50 a 100 µg de proteína total de sonicados de las células infectadas con los distintos baculovirus y utilizando la medición del consumo de oxígeno mediante un electrodo polarográfico (DWa, Hansatech Instruments Ltd, Norfolk, Inglaterra),

mediciones obtenidas por el Dr. Gerard J. Bannenberg según el protocolo descrito en Bannenberg GJ. y col. (2009).

La regioselectividad y estereoespecificidad de la oxigenación de los ácidos grasos fue establecida mediante la identificación de los productos de la oxigenación de las LOXs determinada por cromatografía de gases/ espectrometría de masas (GC-MS) por el Dr. Gerard J. Bannnenberg según el protocolo descrito en Bannenberg GJ. y col. (2009).

3.9. IDENTIFICACIÓN Y CUANTIFICACIÓN DE OXILIPINAS

3.9.1. Identificación de la acumulación de oxilipinas.

Para la determinación del perfil de oxilipinas en tejidos aéreos y tejidos de raíz, se recogieron entre 0,4 a 0,7 g de tejido fresco de plantas de 10 días de edad, se liofilizó y el tejido liofilizado fue analizado en el laboratorio del Prof. Matts Hamberg.

3.9.2. Cuantificación de la producción de 9-HOT en plantas

Para la determinación de los niveles de 9-HOT en tejidos aéreos y tejidos de raíz, se recogieron entre 0,4 a 0,7 g de tejido fresco, por muestra examinada. Una vez congelados los tejidos, se homogenizaron en 10 mL de una solución de hidroxicloruro 0-metilhidroxilamina 30 mM en metanol. Las muestras se enviaron al laboratorio del Profesor Mats Hamberg (Instituto Karolinska, Suecia) para su análisis utilizando como sustrato ácido linolénico.

4. AISLAMIENTO DE LÍNEAS DE ADN-T EN HOMOCIGOSIS

Para genotipar las plantas de la población segregante perteneciente a cada una de las líneas de inserción de ADN-T utilizadas, se preparó ADN genómico de al menos 20 plantas independientes y se procedió a efectuar dos PCR diferentes. Para la primera de ellas se usaron dos cebadores específicos

del gen de interés, mientras que en la segunda, se utilizó un cebador correspondiente al gen que contiene la inserción, y un segundo cebador específico de la secuencia del ADN-T (LBa1 para las líneas del SALK y LB3 para las líneas del SAIL). El análisis de las plantas que contenían la inserción de ADN-T en homocigosis, permitía observar una banda en la segunda PCR y ninguna en la primera (debido a que el tamaño del ADN-T excede la procesividad de la polimerasa). En las plantas en las que la inserción de ADN-T se encontraba en heterocigosis se observaba una banda en ambas PCR, mientras que en las plantas silvestres sólo se observaba una banda en la primera PCR. La información referente a las líneas de inserción para los genes *LOX1* y *LOX5* y los cebadores usados en las reacciones de PCR se muestran en la Tabla 4.

5. GENERACIÓN DE PLANTAS TRANSGÉNICAS

5.1. CONSTRUCCIONES GUS

Se llevaron a cabo técnicas de bilogía molecular estándar para generar las construcciones *LOX1:GUS, LOX2:GUS, LOX3:GUS, LOX4:GUS, LOX5:GUS, LOX6:GUS*, en las que los fragmentos de ADN correspondientes a los promotores de dichos genes, se fusionaron a la región codificante del gen delator *GUS,* presente en el plásmido pBI101.2 (Jefferson y col. 1987). Los fragmentos utilizados se extendían desde las posiciones -973, -1083, -1141, -871, -970 y -1032 de los promotores *LOX1*, *LOX2, LOX3, LOX4, LOX5* y *LOX6*, respectivamente, hasta la posición -1 de cada uno de ellos, designando como +1 al primer nucleótido del ATG de iniciación de la traducción (Tabla 5).

Las construcciones se introdujeron en la estirpe *Agrobacterium tumefaciens* C58C1:pTi (nos+) (Yanofsky y Nester, 1986) y se transfirieron a plantas de *A.thaliana* mediante la transformación siguiendo el método descrito por Bechtold y col. (1998).

Se seleccionaron para los estudios líneas transgénicas homocigotas. A continuación se detallan los métodos requeridos para la obtención de estas plantas transgénicas.

5.2. TRANSFORMACIÓN DE PLANTAS DE *A. THALIANA*

La transformación de *A. thaliana* se realizó usando plantas crecidas en tierra durante tres semanas. Se utilizó el método de inmersión floral con una suspensión de *A. tumefaciens* en medio MS suplementado con 5% de sacarosa, 11 ng/mL de bencil amino purina (BAP) y 0,02% de "Silwet L-77" (Bechtold y col., 1998).

5.3. SELECCIÓN DE LÍNEAS TRANSGÉNICAS

Las semillas transgénicas T1 se sembraron en placas Petri con medio MS suplementado con el antibiótico de selección (kanamicina o higromicina) y claforán (500 mg/L), para evitar el crecimiento de *A. tumefaciens*. Las plántulas resistentes al antibiótico fueron trasplantadas para recoger la generación T2. Las semillas T2 se volvieron a crecer en presencia del antibiótico de selección para determinar la segregación de la resistencia al antibiótico. Las líneas que presentaban una segregación 3:1, esperada para una única inserción de ADN-T en el genoma, fueron seleccionadas y transferidas a tierra para obtener la siguiente generación. Finalmente las semillas T3 se sembraron nuevamente en el antibiótico de selección para identificar las plantas homocigotas, con las que posteriormente se realizaron los distintos estudios de interés.

6. TRATAMIENTOS EN PLANTAS ADULTAS

6.1. INOCULACIÓN DE BACTERIAS FITOPATÓGENAS

6.1.1. Preparación del inóculo

A partir de una placa fresca de *Pseudomonas* se creció un césped en placas con medio King's B suplementado con el antibiótico correspondiente (Km para *Pst* DC3000 y Km-Tc para *Pst* DC3000 *avr*RPM1) y se dejaron crecer durante 24 horas a 28ºC.

Transcurrido ese tiempo se resuspendieron las bacterias en $MgCl_2$ 10mM y por último se ajustó el cultivo a 10^6 ufc/ml, tomando como referencia que 10^7 ufc presentan una absorbancia de 0,1DO irradiando una longitud de onda de 600 nm mediante un espectrofotómetro.

6.1.2. Infiltración de la suspensión bacteriana

Para la inducción de los genes de defensa, así como para el estudio de la respuesta hipersensible, la inoculación de bacterias se realizó inyectando con jeringuillas estériles de 1 mL plastipak (Becton Dickinson) una suspensión de 10^6 ufc/ml de *Pseudomonas syringae pv* tomate DC3000, o *Pseudomonas syringae* pv tomate DC3000 *avr*Rpm1 en el lado abaxial de hojas de rosetas de plantas de 4 semanas de edad.

Los síntomas de infección fueron examinados en hojas infectadas con una suspensión de 10^5 y 10^6 ufc/mL. Para cada genotipo se examinaron un mínimo de 20 plantas en tres experimentos independientes.

6.2. CURVAS DE CRECIMIENTO BACTERIANO

La determinación del crecimiento bacteriano en las hojas de las plantas inoculadas se realizó según el protocolo descrito por Whalen y col. (1991). Para

ello, se inocularon hojas sanas de la roseta basal, de plantas de 4 semanas, con una suspensión bacteriana que contenía 10^5 ufc/mL. A los tiempos apropiados (0, 2 y 4 días), se cortaron discos de 0,6 cm de diámetro de las zonas inoculadas, que se homogeneizaron en agua. Para cada tiempo analizado, se utilizaron 6 muestras independientes conteniendo, cada una de ellas, 3 discos de hojas infectadas, seleccionadas al azar entre las plantas tratadas. La población bacteriana del tejido vegetal se determinó extrayendo en agua las bacterias de los discos obtenidos, posteriormente se realizaron diluciones seriadas de las suspensiones bacterianas obtenidas y finalmente se crecieron éstas en medio sólido KB con los antibióticos de selección correspondientes. Los datos presentados corresponden a las medias de los valores obtenidos en al menos tres experimentos independientes y se reflejan en un gráfico con escala logarítmica.

6.3. TRATAMIENTO CON ROSA DE BENGALA

Para la cuantificación del daño celular producido tras el tratamiento con RB, se inocularon en la superficie del envés foliar de hojas de plantas de 4 semanas de edad con una solución a 10 µM de RB.

7. TRATAMIENTOS EN PLÁNTULAS CRECIDAS *IN VITRO*

7.1. TRATAMIENTO CON OXILIPINAS PARA EL SEGUIMIENTO FENOTÍPICO Y ANÁLISIS HISTOLÓGICOS

Para el análisis de la respuesta fenotípica de las plantas a los distintos productos, se crecieron durante 4 días en medio MS tras los cuales se transfirieron a placas con el mismo medio suplementado con el producto de interés a distintas concentraciones (entre 2 y 75 µM). Las oxilipinas utilizadas fueron proporcionadas por el Prof. Mats Hamberg (Instituto Karolinska, Suecia).

Se siguió este mismo procedimiento para el análisis de las plantas portadoras de la construcción en el que la expresión del gen reportero GUS se encuentra bajo la dirección del promotor artificial de respuesta a etileno (*EBS::GUS*).

7.2. TRATAMIENTO CON COMPUESTOS PARA ANÁLISIS MOLECULARES

El tratamiento de las plantas con 9-HOT y RB, con objeto de recoger tejido y extraer ARN, se realizó aplicando sobre las plántulas, crecidas durante 10 días en la superficie de placas con medio MS, una solución de medio MS 0,5% líquido conteniendo el compuesto de interés. Las concentraciones usadas en estos tratamientos fueron 50 μM para el 9-HOT y 10 μM para el RB. El tejido tratado se recogió a los tiempos indicados en cada caso, posteriormente se congelaron en N_2 líquido, y se mantuvieron a -80°C hasta su utilización.

7.3. TRATAMIENTO CON COMPUESTOS PARA ANÁLISIS MOLECULARES EN MEDIO LÍQUIDO.

Los tratamientos de las plantas con ACC, 9-oxo-C_9, JA, 9KOT y 9-HOT con objeto de recoger tejido y extraer proteínas, se realizaron creciendo las plántulas durante 7 días en medio MS líquido, tras este tiempo, se cambiaban las plantas a medio MS fresco donde se dejaban crecer 24 h más. Posteriormente se realizaron los tratamientos, se recogieron las muestras a analizar, se congelaron en N_2 líquido, y se mantuvieron a -80°C hasta su utilización.

8. MEDIDA DEL DAÑO CELULAR EN RESPUESTA A RB

La cuantificación del daño celular en los tejidos infectados se determinó midiendo la conductividad de los tejidos tratados, utilizando un conductímetro (Basic30, Crisol). En este ensayo se inocularon con una solución 10 μM de RB tres hojas por planta y 8 plantas por cada tiempo examinado. A partir de las hojas inoculadas se obtuvieron discos de 0,6 cm de diámetro (un disco/hoja),

que se sumergieron en 5 mL de agua milliQ (24 discos) y se mantuvieron en agitación a temperatura ambiente durante 6 horas antes de cuantificar la conductividad. Para cada planta y tiempo examinados, se realizaron al menos 3 ensayos independientes y se determinó la media de los datos obtenidos en cada uno de ellos.

9. TÉCNICAS HISTOLÓGICAS

9.1. DETERMINACIÓN HISTOLÓGICA DE LA ACTIVIDAD β-GLUCURONIDASA

La actividad β-glucuronidasa se localizó en los tejidos de interés mediante ensayos histoquímicos. El tejido analizado se sumergió en una solución con TrisHCl pH 7 100 mM, NaCl 50 mM, ferrocianuro potásico 2 mM, 0,5 mg/mL del sustrato ácido 5-bromo-4-cloro-3-indol β-D-glucurónico (X-Gluc) y metanol al 20%, que aumenta la especificidad y reproducibilidad de la tinción. Posteriormente se incubaron a 37°C en oscuridad durante un día. Tras la incubación, las muestras fueron tratadas según el tipo de tejido analizado. En el caso de hojas de 2-4 semanas, éstas fueron sumergidas en etanol 70% hasta eliminar la pigmentación endógena del tejido y posteriormente rehidratadas con agua. En cambio, cuando el ensayo se realizó sobre plántulas de unos 10 días de crecimiento, se llevó a cabo un proceso de clarificación para observar mejor la actividad β-glucuronidasa en las raíces.

9.1.1. Análisis de los primordios radiculares

La clarificación de las raíces para evaluar la expresión de las transgénicas durante el desarrollo de los primordios radiculares se realizó según el protocolo descrito por Malamy y Benfey (1997). Las plántulas se transfirieron a una placa Petri conteniendo HCl 0,24N en 20% de metanol y se incubaron en un bloque de calor a 57°C durante 15 minutos. Esta solución fue remplazada con NaOH 7% en 60% de etanol durante 15 minutos a temperatura ambiente. Las raíces después fueron rehidratadas durante 5 minutos en etanol del 40, 20 y 10% y

sometidas dos veces a vacío durante 7 minutos en una solución de etanol 5% y glicerol 25%. Las raíces fueron montadas en glicerol 50% y se visualizaron en el microscopio con un contraste Nomarski. Para la cuantificación del número de primordios radiculares se utilizaron unas 20 plantas por cada experimento y por cada una de las líneas examinadas.

9.2. TINCIÓN DE CALOSA

La calosa se identifica mediante la fluorescencia inducida por luz ultravioleta del fluorocromo del producto azul de anilina (Stone y col., 1985). Para proceder a su visualización, las plántulas se sumergieron en una solución de azul de anilina 0,01% (Sirofluor; Biosupplies) durante 30 minutos. Después de lavarlas con agua, se montaron en glicerol al 50% para su visualización en el microscopio con luz ultravioleta. En el caso de la tinción de hojas maduras, éstas se destiñeron previamente con etanol 100% durante 2 días y se rehidrataron con agua antes de teñir.

9.3. TINCIÓN DE ESPECIES REACTIVAS DE OXÍGENO (ROS)

Las plántulas fueron sumergidas en una solución de NBT (azul nitro-tetrazolio cloruro, Promega) 0,5mg/ml en fosfato potásico 0,1M pH7 durante 30 minutos, en la misma placa de cultivo donde fueron crecidas, para evitar cualquier tipo de daño en la raíz (Carol y col., 2005). Posteriormente se retiró la solución de NBT y se añadió etanol 100% para detener la reacción. Las plántulas se montaron en MS 0,5% líquido y fueron visualizadas al microscopio con un filtro Nomarski.

9.4. TINCIÓN DE AGUA OXIGENADA

La producción de H_2O_2 se examinó en hojas infectadas 48 h después de la inoculación con las distintas cepas bacterianas analizadas. La acumulación de H_2O_2 se visualizó mediante la tinción con 3,3'-diaminobencidina tetracloruro (DAB) (Sigma-D-8001), según se describe en Thordal-Christensen y col. (1997).

Las hojas se sumergieron en una solución de 1 mg/mL pH 3,8 de DAB durante unas 12h a 25ºC, tras lo cual se decoloraron con etanol al 96%.

9.5. TINCIÓN DE MUERTE CELULAR

La muerte celular producida durante las infecciones bacterianas se tiñó con una solución de Azul de tripano (10 mL de ácido láctico al 85%, 10 mL de glicerol 98%, 10 mL de agua destilada y 20 mg de azul de tripano). Las hojas se hirvieron en un microondas durante 5 segundos y se clarificaron con una solución de cloral hidrato (50 g en 20 mL de agua destilada).

10. APLICACIONES BIOINFORMÁTICAS

. Los programas informáticos o bases de datos utilizados han sido:

TAIR, The Arabidopsis Information Resource (http://www.arabidopsis.org/): base de datos que nos ha proporcionado información sobre el genoma de Arabidopsis.

Chromas y 4Peaks: para el análisis de secuencias.

ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html): utilizado para realizar alineamientos de secuencias.

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi): utilizado para comparar tanto secuencias de nucleótidos como de aminoácidos en la base de datos de NCBI.

ADN-T express (http://signal.salk.edu/cgi-bin/tdnaexpress): para la búsqueda de mutantes de inserción de ADN-T en los genes de interés (Alonso y col., 2003).

Genevestigator (https://www.genevestigator.ethz.ch): utilizado para buscar patrones de expresión de los genes analizados en este trabajo. Está basado en los datos obtenidos mediante micromatrices de oligonucleótidos (Zimmermann y col., 2004).

dCAPS Finder (http://helix.wustl.edu/dcaps/dcaps.html): utilizado para buscar dianas de restricción polimórficas entre dos secuencias.

TABLAS

Tabla 1. Información de los antibióticos y las respectivas concentraciones usadas en este trabajo.

Agente Selectivo	Método de selección	Solución Madre	Disolvente	Concentración final
ampicilina	resistencia	50 mg/ml	H_2O	50µg/ml
kanamicina	resistencia	25 mg/ml	H_2O	25 µg/ml
rifampicina	resistencia	25 mg/ml	DMSO	100 µg/ml
tetraciclina	resistencia	5 mg/ml	H_2O	10 µg/ml
actividad β-galactosidasa	coloración colonias			

Tabla 2. Cebadores específicos utilizados para analizar la expresión génica por RT-PCR

locus	GEN	Secuencia de cebadores	Longitud Amplicón
At1g55020	*LOX1*	5'-TTTGGATGGACTCACTGTTGAAG-3' 5'-GTCCCAAGTATGGCATCAGTGT-3'	89 pb
At3g22400	*LOX5*	5'-GGCTGGAGGACTGACGAA-3' 5'-TTTGCAGAGTCCAGACAGCTC-3'	111 pb

Amplicón	3' cebador	Posición cebador 3'	5' cebador	Posición cebador 5'	Gen	*locus*
2624	GAAGCGAGAGTTGTTTCAGATAG	+2595	GAATCAAAACTAGTACTTCACCCAA	-29	*LOX1*	At1g55020
2727	TGAGAAAGAAAATGCATAGATC	+2711	AGAAGAGAAAACTAGTATGTATTGTAG	-16	*LOX2*	At3g45140
2772	CCTTCTTAATTATATAGATACACTATTAG	+2769	GTGATGGCCTTAGCTAAAGAGTTA	-3	*LOX3*	At1g17420
2808	ATTGAATAATTCTAAATAGATACACTAT	+2792	ACTAATTCTTACTAGTATGGCTTTAG	-16	*LOX4*	At1g72520
2689	GAGAGAGGGTTTATCTTTAGATTGAGAC	+2677	ACTAGTATCGCCATGATCCACACCG	-12	*LOX5*	At3g22400
2772	CATCTAAATGGAAATGCTGTTTGG	+2757	CAAACTAGTGTAAAAATGTTCGTAGCA	-15	*LOX6*	At1g67560

Tabla 3. Información de los fragmentos de los ADNc clonados en las líneas de células de insecto generadas y los cebadores usados para amplificar dichos fragmentos.

Tabla 4. Información de las líneas de ADN-T de los genes *lox1-1* y *lox5-1* y los cebadores usados para identificar las inserciones.

Locus *Gen*	Línea de inserción	Localización de la inserción	Secuencia de cebadores 5'….3'	Longitud Amplicón
At1g55020 *LOX1*	SALK_059431	Exón 8	CAAACCGACACAGAGCTCCAAGCC' / CCCTGCCGGTGACTCCGCCTTCAC	576 pb
At3g22400 *LOX5*	SALK_044826	Intrón 2	GAATCAAAACTAGTACTTCACCCAA / GAAGAAGACTAGTACAATGGAGGAAGAT	111 pb

Tabla 5. Información de los fragmentos de los promotores clonados en las líneas transgénicas generadas y los cebadores usados para amplificar dichos fragmentos.

Transgénicas	Secuencia de los cebadores 5'…3'	Posición del Fragmento	Longitud
35S::LOX1:GUS	GTACGGTGAAGCTTCTCTGCCGC / GGGTGAAGCTTGAAGTTTTGATTCAC	-973 a -1	972
35S::LOX2:GUS	GCGTCCTCTAGAGATTGAGAGAGAC / CTCTTCTAGAAGGCTTACATTCCTC	-1083 a -1	1082
35S::LOX3:GUS	AAGCTTCGCGTCGTGTCAGACTCG / TCTCGCGTGGTATACTATTAGTGAAG	-1141 a -1	1140
35S::LOX4:GUS	AAGCTTGCACTATCCGACATGATC / GTTGAAGCTTCAAGACTGAG	-871 a -1	870
35S::LOX5:GUS	AAGCTTGCTATGTACGATACATATGC / CTGCGATGTCGGTGTGGATCCTGGCG	-970 a -1	969
35S::LOX6:GUS	CGATTGGCGAGATGAAAAAGCTTAG / GTTTGAAGCTTGGCAGTGTGAAGAG	-1032 a -1	1031

RESULTADOS

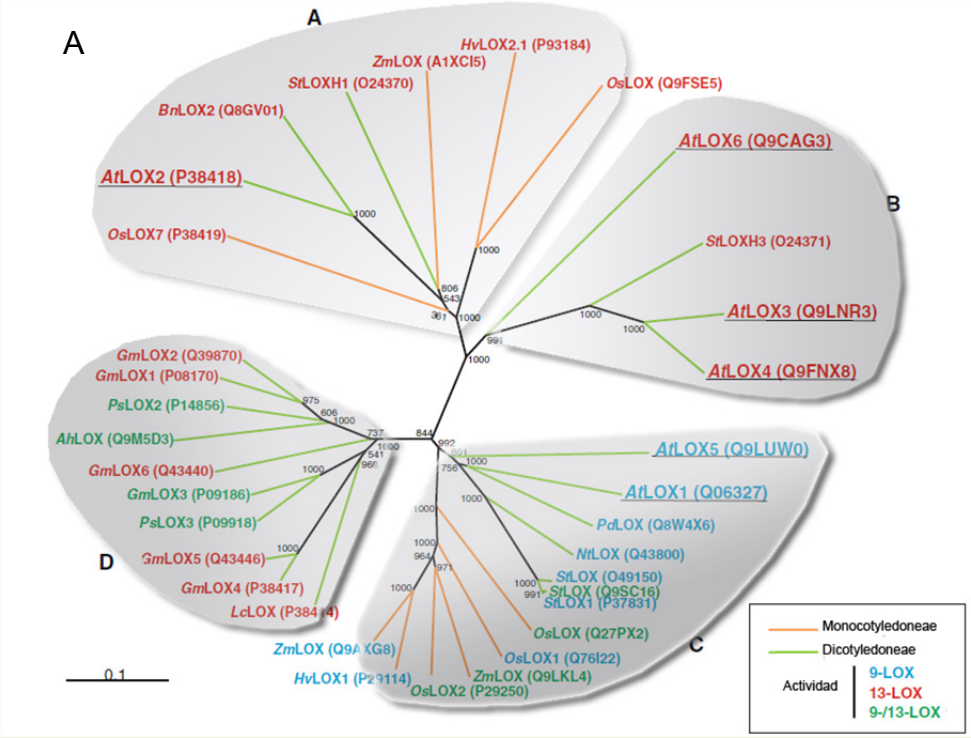
RESULTADOS

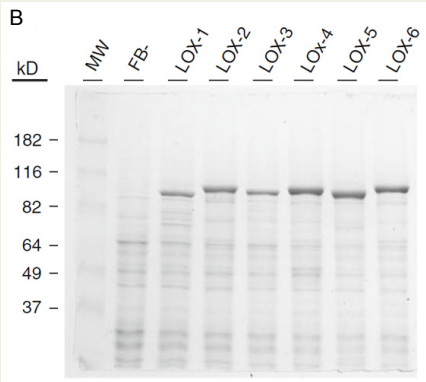
1. IDENTIFICACIÓN Y CARACTERIZACIÓN DE LIPOXIGENASAS EN PLANTAS DE *ARABIDOPSIS*

1.1. IDENTIFICACIÓN GÉNICA Y DETERMINACIÓN DE LA ACTIVIDAD ENZIMÁTICA

Los estudios realizados con objeto de caracterizar la actividad de las enzimas lipoxigenasa (LOX) en plantas, han demostrado su acción como oxigenasas de ácidos grasos poliinsaturados, mayoritariamente linolénico (18:3) y linoleico (18:2), catalizando la incorporación de oxígeno en el carbono 9 ó 13 de sus correspondientes substratos. Además, estos estudios han demostrado la presencia de ambas actividades enzimáticas, designadas 9-LOX y 13-LOX, en una misma especie vegetal, en la que inician rutas de síntesis específicas, a través de las que se generan derivados lipídicos distintos (Vick y Zimmerman, 1983; Grechkin y col., 1995; Liechti y Farmer, 2006). En base a estos resultados y dado nuestro interés en el estudio de las enzimas 9-LOX, se propuso identificar los genes que codifican dichas proteínas en plantas de *Arabidopsis thaliana*.

El análisis de la secuencia genómica de *Arabidopsis* revelaba la presencia de seis genes *LOX* candidatos, designados *LOX1-LOX6*, cuya expresión y traducción podrían dar lugar a proteínas con actividad lipoxigenasa. El estudio filogenético de las secuencias proteicas predichas, junto con las correspondientes a 28 lipoxigenasas de actividad conocida, procedentes de otras especies vegetales, permitía dibujar un árbol filogenético en el que las proteínas examinadas aparecían distribuidas en cuatro grupos principales, designados A-D (Figura 1A). Las proteínas contenidas en los grupos A y B, correspondían en todos los casos a enzimas con actividad 13-LOX, y en ellos se encontraban cuatro de las secuencias de *Arabidopsis*, LOX2, LOX3, LOX4 y LOX6, que debían corresponder, por lo tanto, a enzimas con actividad 13-LOX. Las dos proteínas de *Arabidopsis* restantes, LOX1 y LOX5, aparecían contenidas dentro del grupo C, en el que se detectaba la presencia de enzimas con actividad 9-LOX, así como de enzimas con capacidad para catalizar ambas actividades enzimáticas, 9-LOX y 13-LOX. Estos resultados indicaban que las proteínas LOX1 y LOX5 podían corresponder a enzimas con actividad 9-LOX exclusivamente, o a enzimas con actividad dual 9-LOX y 13-LOX, por lo que, para distinguir entre estas dos posibilidades se procedió a examinar experimentalmente la actividad de cada una de las enzimas lipoxigenasas identificadas en *Arabidopsis*. De esta manera, y tras expresar en células de insectos los ADNc correspondientes a los seis genes *LOX* identificados (Figura1 B y C), se procedió a caracterizar los productos generados por cada una de las enzimas examinadas mediante HPLC y espectrometría de masas. Así, utilizando el ácido linoleico como sustrato y los ésteres metilados correspondientes (13-HOD-E,Z, 13-HOD-E,E, 9-HOD-E,Z y 9-HOD-E,E) como estándares, se pudo determinar que de las seis proteínas LOX codificadas en el genoma de *Arabidopsis*, las enzimas LOX1 y LOX5 poseían actividad 9-LOX, que las cuatro proteínas restantes, LOX2, LOX3, LOX4 y LOX6, poseían actividad 13-LOX, y que ninguna de ellas poseía actividad dual 9-LOX /13-LOX.





C

Enzima	Sustrato	Ácido hidróxido	Actividad enzimática
LOX1	C18:2	9*S*-hydroxy-18:2	9*S*-LOX
LOX2	C18:2	13*S*-hydroxy-18:2	13*S*-LOX
LOX3	C18:2	13*S*-hydroxy-18:2	13*S*-LOX
LOX4	C18:2	13*S*-hydroxy-18:2	13*S*-LOX
LOX5	C18:2	9*S*-hydroxy-18:2	9*S*-LOX
LOX6	C18:2	13*S*-hydroxy-18:2	13*S*-LOX

Figura 1 Identificación y caracterización de los enzimas de lipoxigenasas de *A.thaliana*.
(A) Relación filogenética entre lipoxigenasas de *A.thaliana* y lipoxigenasas enzimáticamente caracterizadas de otras especies de plantas. El árbol filogenético de las proteínas LOX se llevó a cabo mediante los métodos de parsimonia y de distancias. Las ramas en verde y naranja representan lipoxigenasas de especies monocotiledóneas y dicotiledóneas, respectivamente. Las 9-LOXs están marcadas en azul, las 13-LOXs en rojo y las LOXs con especificidad dual en verde. Las 6 LOXs de *A.thaliana* aparecen subrayadas. La identidad de cada LOX está indicada por su número de Uniprot (http://www.pir.uniprot.org). La escala corresponde a una distancia de 10 cambios por cada 100 aminoácidos.

(B) Expresión heteróloga de LOX de *A.thaliana* en células de insecto. Se muestra un gel de SDS-poliacrilamida con la expresión de las LOXs de *A.thaliana* en células de insecto High Five infectadas con baculovirus recombinantes. Cada línea corresponde a lisados derivados de 125,000 células aproximadamente.

(C) Diagnóstico de los iones generados por cada una de las LOXs de *A.thaliana*. Se muestran los ácidos hidroxi-octadecadienóicos derivados del ácido linolénico (18:2) generados por las proteínas examinadas e identificados mediante análisis de HPLC y espectrometría de gases

1.2. ANÁLISIS DE LA EXPRESIÓN DE LOS GENES *LOXS* DE *ARABIDOPSIS*.

Para profundizar en la caracterización de los genes 9-LOX identificados, se procedió a examinar su patrón de expresión, generando para ello, genes quimera en los que los promotores de los genes *LOX1* y *LOX5* de interés, se fusionaron a la secuencia del gen marcador *β-glucuronidasa* (*GUS*).

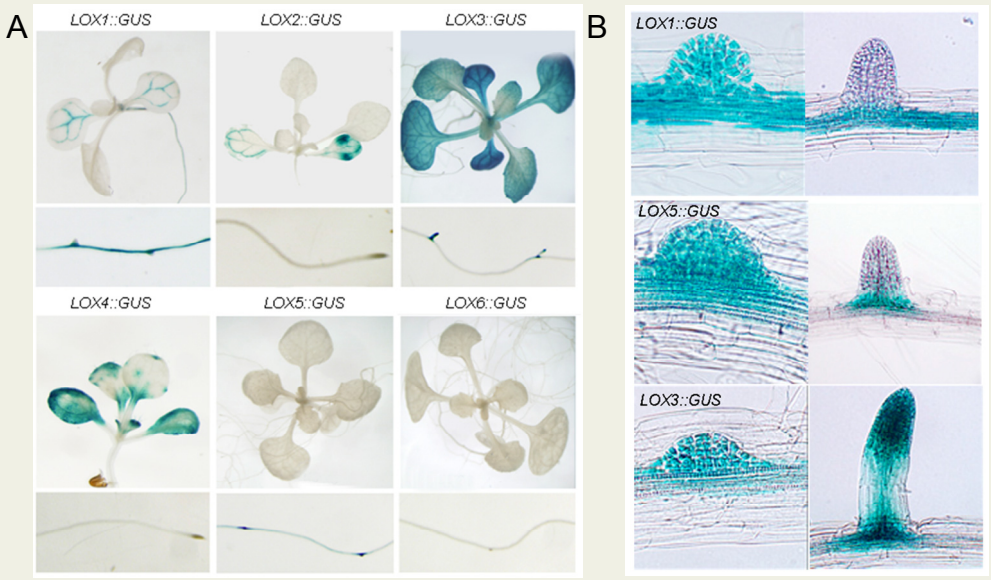


Figura 2. Análisis de la expresión de las lipoxigenasas en plantas sanas de *Arabidopsis*.

Ensayo histoquímico tipo GUS en plantas transgénicas portadoras de construcciones quiméricas en las que los promotores de cada una de las lipoxigenasas de *Arabidopsis* se fusionaron al gen de la *β-glucuronidasa*.

(A) Se muestran imágenes representativas de la parte aérea y de las raíces de plantas crecidas durante 10 días en medio MS.

(B) Detalle de primordios radiculares representativos de plántulas transgénicas portadoras de las construcciones *LOX1::GUS, LOX5::GUS* y *LOX3::GUS*.

Dichas construcciones, designadas *LOX1::GUS* y *LOX5::GUS*, se introdujeron en plantas silvestres de *Arabidopsis* mediante transformación, y se procedió a seleccionar líneas homocigotas en las que se examinó la localización de la actividad GUS en tejidos de hoja y raíz de plantas transgénicas crecidas *in vitro*. De forma paralela, se procedió a generar construcciones quimera conteniendo los promotores de los genes 13-LOX (*LOX2, LOX3, LOX4 y LOX6*) fusionados al gen *β-glucuronidasa* (*GUS*) con las que se generaron (ver materiales y métodos para una descripción detallada de las construcciones), igualmente, plantas transgénicas (*LOX2::GUS, LOX3::GUS, LOX4::GUS, LOX6::GUS*), en las que se examinó la actividad GUS dirigida por los promotores correspondientes. El ensayo de tinción histoquímica de actividad GUS permitió identificar los órganos y tejidos de la planta en los que se expresan cada uno de los genes examinados (Figura 2A y 2B).

Los resultados de estos análisis revelaron que la expresión *LOX1::GUS* se detectaba en los haces vasculares de hojas y raíces, así como en las células correspondientes a los primordios radiculares, mientras que la expresión *LOX5::GUS* aparecía localizada exclusivamente en los primordios radiculares (Figura 2A). Por otro lado, el análisis de los genes *13-LOXs*, reveló que la actividad GUS dirigida por los promotores *LOX2* y *LOX4*, se localizaba exclusivamente en las hojas de la planta, mientras que la correspondiente al gen *LOX3* aparecía en hojas y en primordios radiculares. Finalmente, el análisis de las plantas transgénicas *LOX6::GUS* no permitió detectar actividad GUS en ninguno de los tejidos examinados (Figura 2A).

Los patrones de expresión descritos se mantuvieron durante el desarrollo de las plántulas. Sin embargo, cabe mencionar que tras la emergencia de las raíces laterales, la expresión *LOX1::GUS* y *LOX5::GUS* aparecía limitada a las células situadas en la base de dichas estructuras, mientras que en el caso de la construcción *LOX3::GUS* la actividad GUS aparecía tanto en la base, como en los meristemos radiculares localizados en la zona apical de las raíces laterales (Figura 2B).

1.3. ANÁLISIS BIOQUÍMICO DE LA ACTIVIDAD LIPOXIGENASA EN PLÁNTULAS DE *ARABIDOPSIS*

Los resultados derivados del análisis de la expresión de los genes *LOX*s en plantas sanas, reflejaban una mayor contribución de las enzimas 9-LOX en los tejidos de raíz, y de las 13-LOX en los tejidos de hojas. Con objeto de determinar si los patrones de expresión caracterizados estaban acompañados de la producción y acumulación de oxilipinas, se procedió a caracterizar los derivados 9-LOX y 13-LOX que se acumulaban en dichos tejidos.

Compuesto	Origen	Ion	Tiempo de retención	Raíces	Hojas
9-HOT	9-LOX	223. 311	13.09	+	+
9-KOT	9-LOX	306. 237. 185	13.58-14.32	+	+
9,10-Epoxi-13-OH	9-LOX	327. 239	14.59-14.92	+	+
2-Hidroxi-9-KOT	α-DOX +9-LOX	394. 335	15.60	+	+
13-HOT	13-LOX	311	13.03	-	+

Tabla 6. Detección de oxilipinas en raíces y hojas de plantas de *Arabidopsis*.
Se obtuvo 0,4-0,7 g de tejido de raíz y la misma cantidad de parte aérea de plantas de 10 días de edad y tras la liofilización fue analizado mediante GC/MS.

Los análisis bioquímicos realizados a este objeto permitieron determinar que distintas oxilipinas derivadas de la actividad 9-LOX estaban presentes en los tejidos de raíces y hojas de plantas sanas, mientras que derivados de las 13-LOXs se detectaban únicamente en las hojas (Tabla 6). Estos análisis revelaron, además, la presencia de oxilipinas derivadas de la actividad de α-dioxigenasas en los tejidos de la raíz, así como derivados (2-Hidroxi-9-KOT) para cuya síntesis se requiere la participación de las actividades enzimáticas 9-LOX y α-DOX.

Los resultados de estos análisis demuestran una contribución preferente de la actividad 9-LOX, y de sus derivados, en los tejidos de raíz.

1.4. ANÁLISIS DE LA EXPRESIÓN DE 9-LIPOXIGENASAS EN RESPUESTA A PATÓGENOS

Una vez caracterizado el patrón de expresión de los genes 9-lipoxigenasa en plántulas sanas, se procedió a examinar su posible inducción durante la respuesta de la planta a la infección de bacterias hemibiotrofas del género *Pseudomonas*. De esta manera, y al igual que ocurre en plantas de tabaco (Rusterucci y col., 1999), se propuso evaluar el papel de esta ruta de síntesis de oxilipinas durante la infección de este tipo de patógenos, y a determinar si su activación contribuye a la defensa de la planta.

Para realizar este estudio se procedió a examinar mediante northern blot los niveles de expresión de los genes *LOX1* y *LOX5* en hojas inoculadas con las cepas *Pst* DC3000 (virulenta) y *Pst* DC3000 *avr*RPM1 (avirulenta) que establecen en plantas de *Arabidopsis* una interacción compatible e incompatible, respectivamente. La proteína *avr*RPM1 que se produce en la cepa avirulenta se libera, a través del sistema de secreción tipo III de la bacteria, al citoplasma de la célula vegetal, en donde, a través de su interacción con el producto del gen de resistencia *RPM1* activa una reacción hipersensible de defensa vegetal, que protege a la planta de la infección limitando el crecimiento bacteriano y su progresión en la planta.

El análisis de los transcritos derivados de los genes *LOX1* y *LOX5* en las plantas infectadas revelaron la activación de su expresión en respuesta a ambas cepas bacterianas. La inducción del gen *LOX5* se observó en las muestras de ARN recogidas a las 4 y 8 horas tras inoculación de la bacteria *Pst DC3000 avr*RPM1, mientras que la expresión del gen *LOX1* se observó, inicialmente en las muestras recogidas a las 8 horas de la inoculación, y se mantuvo hasta el último tiempo examinado (72 horas). La expresión de los dos genes examinados se indujo igualmente tras la infección de la cepa virulenta, aunque su activación

se retrasó en el tiempo con respecto a la observada en respuesta a la cepa avirulenta.

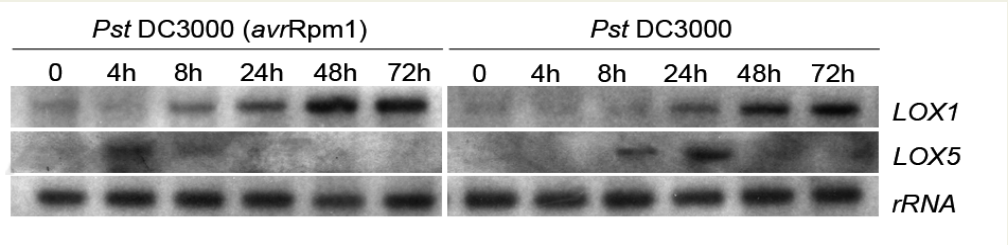


Figura 3. Análisis de la expresión de los genes 9-LOXs en infecciones bacterianas.
Cinética de la acumulación de los transcritos de los genes 9-LOXs en hojas de plantas silvestres de *Arabidopsis* de 4 semanas de edad inoculadas con las cepas *Pst* DC3000 *avr*Rpm1 (10^6 ufc/mL) y *Pst* DC3000 (10^6 ufc/mL). Los filtros conteniendo los ARNs obtenidos a distintos tiempos de infección se hibridaron con sondas preparadas a partir de los genes *LOX1* y *LOX5*.

Los datos descritos revelaban que la expresión de los dos genes 9-LOX caracterizados se inducía en respuesta a una infección patogénica y, por tanto, que los compuestos sintetizados por la acción de las enzimas 9-LOXs, forman parte de la respuesta de la planta frente a la presencia de bacterias patógenas.

1.5. PAPEL DE LAS ENZIMAS 9-LOXS EN LA RESPUESTA DE LA PLANTA FRENTE A LA INFECCIÓN DE BACTERIAS HEMIBIOTROFAS

Con objeto de examinar la participación de las enzimas 9-LOX en la respuesta de la planta frente a la infección de bacterias hemibiotrofas, se procedió a generar plantas mutantes, carentes de dicha actividad, y a caracterizar esta respuesta en dichas plantas en relación a la observada en plantas silvestres. Para ello, se obtuvieron mutantes individuales, designados como *lox1* y *lox5*, conteniendo una inserción de ADN-T en cada uno de los dos genes *LOX1* y *LOX5* de *Arabidopsis*, así como un mutante doble *lox1 lox5*, carente de actividad 9-LOX, que se generó mediante cruzamiento de los mutantes individuales.

La selección de los mutantes descritos se realizó mediante PCR utilizando como molde el ADN genómico extraído de cada uno de ellos, y usando cebadores específicos (mostrados en la Tabla 3) para cada uno de los genes *LOX1* y *LOX5*.

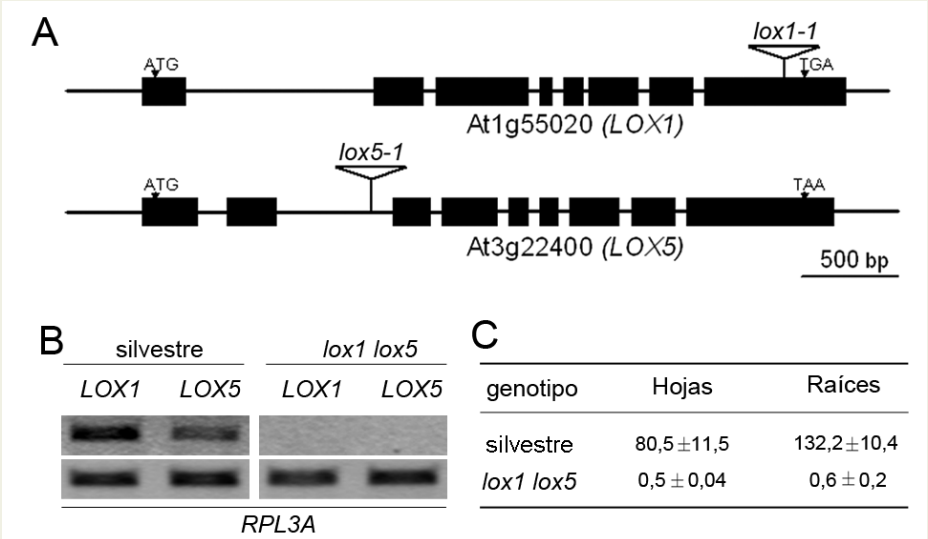


Figura 4. Obtención y caracterización del mutante *lox1 lox5*.

(A) Representación esquemática de las estructuras genómicas correspondientes a los genes *LOX1* y *LOX5*. Los exones están representados como cajas negras. Se indica la posición de las inserciones de ADN-T.

(B) RT-PCR semicuantitativa realizadas sobre muestras de ARN obtenidas a partir de hojas de plantas silvestres y de los mutantes *lox1 lox5*. El nivel de acumulación de los transcritos derivados del gen At1g43170 fue utilizado como control en estos experimentos

(C) Niveles de 9-HOT (nmol g^{-1}) en homogenizados de raíces y de hojas de plantas del tipo silvestre y de *lox1 lox5* incubadas con ácido linolénico.

La validez de los mutantes seleccionados se confirmó mediante RT-PCR, en donde se comprobó la ausencia de los transcritos correspondientes utilizando para ello el ARN obtenido de las plantas mutantes y los cebadores correspondientes (Tabla 2). La representación esquemática de las estructuras genómicas correspondientes a los genes *LOX1* y *LOX5* aparece recogida en la Figura 4A, en la que se indica la posición de los ADN-T insertados en cada uno

de ellos, así como la ausencia de transcritos *LOX1* y *LOX5* en el doble mutante *lox1 lox5* de interés (Figura 4B). Finalmente, la validez del mutante *lox1 lox5* generado se examinó mediante determinación de la actividad 9-LOX en los tejidos de raíz y hoja de las plantas seleccionadas. Para ello, se prepararon homogeneizados a partir de plantas mutantes y silvestres, en los que se procedió a determinar, mediante análisis de GC-MS, la producción de 9-HOT, tras la adición de ácido linolénico a las muestras examinadas.

Los resultados de estos análisis permitieron observar que, de acuerdo a los análisis de expresión mostrados en las Figuras 2A y 2B, la actividad 9-LOX en las plantas silvestres es mayor en el tejido de raíz que en las hojas de la planta, y que dicha actividad disminuye drásticamente en el doble mutante *lox1 lox5* en el que los niveles de 9-HOT están próximos a cero (Figura 4C).

Una vez confirmada la validez de los mutantes *lox1 lox5* generados, se procedió a examinar la respuesta de dichas plantas a la infección de las dos cepas de *Pst* DC3000, virulenta y avirulenta, utilizadas en este estudio. Para ello se inocularon hojas de plantas de 4 semanas de edad con las dos cepas de interés, *Pst* DC3000 *avr*RPM1 y *Pst* DC3000, y se procedió a cuantificar el crecimiento bacteriano en los tejidos infectados a las 72 h horas post tratamiento. Además, se estudió el progreso de la infección mediante northern blot, para ello se analizó la expresión de dos genes utilizados habitualmente en la bibliografía como marcadores de respuesta a estas bacterias, *PR1* y *PR2*, y dos genes cuya expresión se induce en respuesta a 9-HOT, *ABC* y *POX*.

Los resultados de estos análisis no mostraron diferencias significativas en la tasa de crecimiento bacteriano alcanzado por la cepa avirulenta (*Pst* DC3000 *avr*RPM1) entre plantas mutantes *lox1 lox5* y plantas silvestres (Figura 5A). Sin embargo, en el caso de la estirpe virulenta (*Pst* DC3000), los resultados obtenidos muestran que el crecimiento de esta cepa bacteriana alcanza un valor de hasta 10 veces mayor en las hojas de las plantas mutantes *lox1 lox5* que en las de las plantas control (Figura 5A).

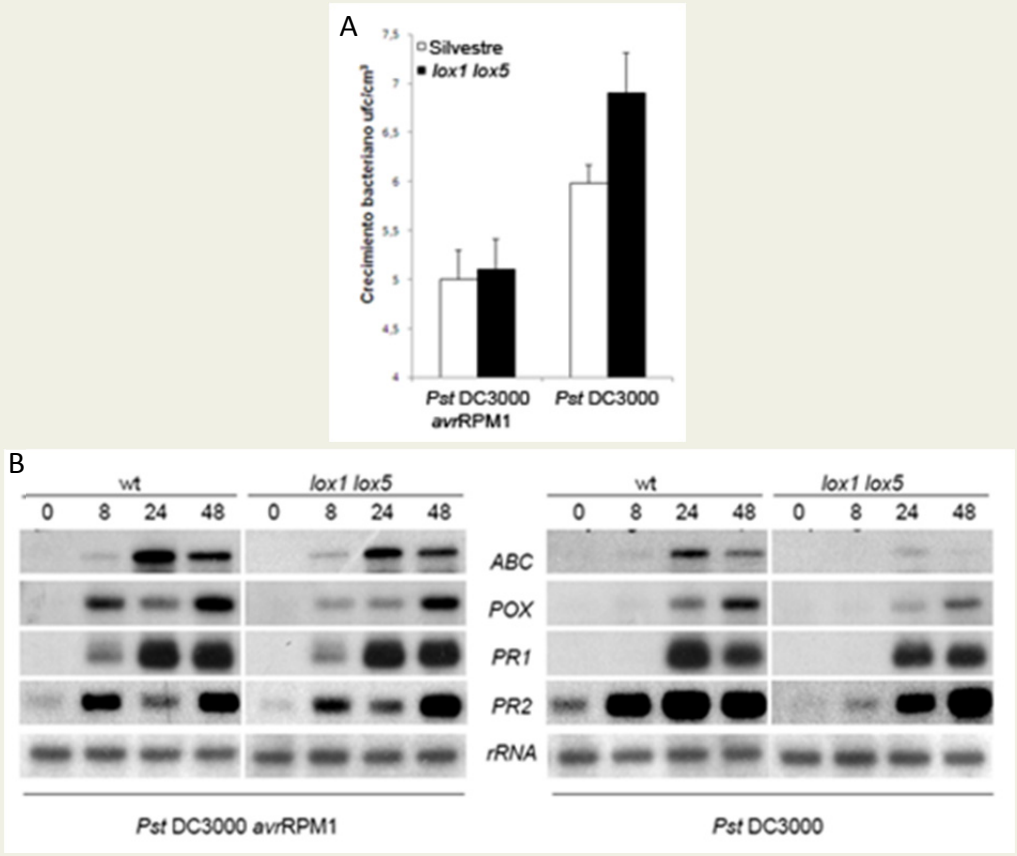


Figura 5. Análisis de plantas silvestres y del mutante *lox1 lox5* en respuesta a infecciones bacterianas.

(A) Nivel de crecimiento alcanzado por la cepa *Pst* DC3000 *avr*RPM1 (10^5 ufc mL^{-1}) y de *Pst* DC3000 (10^5 ufc mL^{-1}) en plantas del tipo silvestre y del mutante *lox1 lox5* a los 4 días de infección. Los datos corresponden a la media y errores estándar de 3 experimentos independientes. (B) Cinética de la acumulación de transcritos de genes de respuesta a 9-HOT y genes marcadores de respuesta a patógenos hemibiotrofos. La expresión de los genes se analizó en hojas de plantas de 4 semanas de edad del tipo silvestres y del mutante *lox1 lox5* inoculadas con las cepas Pst DC3000 *avr*Rpm1 (10^6 ufc/mL) y Pst DC3000 (10^6 ufc/mL). Los filtros conteniendo los ARNs obtenidos a distintos tiempos de infección se hibridaron con sondas preparadas a partir de los genes *ABC, POX, PR1* y *PR2*.

De acuerdo a estos resultados, el análisis de los patrones de acumulación de los ARNs procedentes de los genes examinados permite comprobar un clara disminución en los niveles de transcritos durante la respuesta del mutante *lox1 lox5* a la bacteria PstDC3000 en comparación a los observados en las plantas

control, mientras que dichas diferencias son menos acusadas durante la respuesta de ambos tipos de plantas a la infección de la cepa avirulenta.

Estos resultados ponen de manifiesto el papel de las enzimas 9-LOX en los mecanismos de defensa de la planta frente a la infección de patógenos virulentos, en donde los derivados lipídicos producidos por la acción de estas enzimas contribuyen a limitar el crecimiento del patógeno en la planta.

2. AISLAMIENTO DE MUTANTES INSENSIBLES A 9-HOT

Una vez establecido que los compuestos derivados de la acción de las enzimas 9-LOX tienen un papel positivo en la defensa de la planta frente a la infección de bacterias virulentas, se procedió a examinar los mecanismos de señalización por los que las oxilipinas producidas por la acción de estas enzimas ejercen su acción defensiva.

Resultados previos con plántulas de *Arabidopsis* crecidas *in vitro* en presencia de oxilipinas, habían puesto de manifiesto que la aplicación exógena de oxilipinas producía importantes alteraciones fenotípicas en el desarrollo radicular. Así, entre las oxilipinas testadas 10 de ellas provocaban un crecimiento ondulado denominado *waving*, cinco oxilipinas provocaban una parada del crecimiento radicular con pérdida de la dominancia apical, y dos de ellas producían una reducción generalizada del crecimiento de la raíz. Ensayos posteriores demostraron, además, que los tres fenotipos descritos se inducían a través de rutas de señalización independientes lo que demostraba la especialización funcional de las oxilipinas en relación a su estructura molecular (Vellosillo y col., 2007).

Dentro de las 10 oxilipinas identificadas, el ácido 9-hidroxi-trioneico (9-HOT) era el más activo, provocando un crecimiento ondular homogéneo y reproducible (Figura 6). Esta alteración fenotípica ofrecía la posibilidad de utilizar esta respuesta como una herramienta sencilla para seleccionar plantas mutantes

insensibles a la aplicación de esta oxilipina, y por tanto alteradas en la percepción y/o en los procesos de señalización que determinan la acción de las oxilipinas. La selección de este compuesto para el escrutinio de mutantes estaba basada en primer lugar en que corresponde a un derivado 9-LOX, y por tanto de la ruta de oxilipinas cuya función en la planta se propuso investigar, y en segundo lugar en que los análisis bioquímicos realizados en plantas de *Arabidopsis* (Figura 4C) así como los realizados anteriormente en plantas de tabaco (Rusterucci y col., 1999), habían puesto de manifiesto que dicha oxilipina se acumula en los tejidos de raíz de plantas sanas, y que su producción y acumulación aumenta en tejidos de hoja en respuesta a una infección bacteriana.

La búsqueda de plantas insensibles a la aplicación de 9-HOT, dentro de una población de semillas de *Arabidopsis* mutadas con EMS, permitió seleccionar un elevado número de plantas mutantes que se denominaron *noxy*, del inglés non-responding to oxylipins. Dos de dichos mutantes, *noxy6* y *noxy22*, han sido caracterizados a lo largo de este trabajo.

2.1. CARACTERIZACIÓN FENOTÍPICA DE LOS MUTANTES *NOXY6* Y *NOXY22*

El crecimiento de los mutantes *noxy6* y *noxy22* en placas con medio MS en posición vertical permitió comprobar que la longitud radicular de los dos mutantes examinados es menor que la correspondiente a la de las plantas silvestres. Además y de acuerdo al sistema de selección empleado, los mutantes *noxy6* y *noxy22* no mostraban el fenotipo de ondulación radicular observado en plántulas control crecidas en presencia de 9-HOT (25 µM) (Figura 6).

En esta caracterización inicial se procedió a examinar, igualmente, el comportamiento de los mutantes *noxy6* y *noxy22* en respuesta al tratamiento con las oxilipinas que causaban las alteraciones en el desarrollo radicular descritas anteriormente. A este objeto se examinó el fenotipo radicular de los mutantes *noxy6* y *noxy22* frente a la presencia de 9-Oxo-C_9, JA y OPDA, que provocan la

parada del crecimiento radicular a través de rutas de señalización distintas de las activadas en respuesta a 9-HOT.

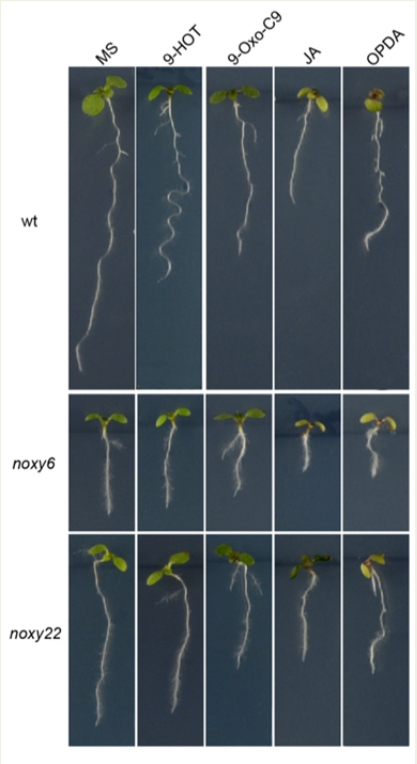


Figura 6. Análisis de las alteraciones fenotípicas producidas en plántulas silvestres, y en los mutantes *noxy6* y *noxy22* crecidas en presencia de oxilipinas.
Imágenes representativas de plantas silvestres, *noxy6* y *noxy22* crecidas durante 3 días en medio MS y transferidas, para continuar su crecimiento, a medios MS fresco o medios MS fresco conteniendo: 25 µM de 9-HOT, 25 µM de 9-Oxo-C_9, 10 µM de JA o 25 µM de OPDA.

Los resultados de estos análisis mostraban que la presencia de 9-Oxo-C_9 provocaba un acortamiento de la longitud de la raíz acompañado de formación de raíces adventicias, y que esta respuesta se manifestaba tanto en plantas silvestres como en los mutantes *noxy6* y *noxy22*. De la misma manera, la presencia de JA y OPDA provocaba el acortamiento de la longitud radicular, tanto en plantas silvestres como en los mutantes examinados. Estos resultados permitían concluir que las mutaciones *noxy6* y *noxy22* afectan preferentemente a la ruta de señalización activada en respuesta a la presencia de 9-HOT y que por tanto la caracterización de dichos mutantes permitirá identificar dos de los componentes moleculares que participan en la señalización de esta respuesta.

2.2. CARACTERIZACIÓN MOLECULAR DE LA RESPUESTA A 9-HOT

Estudios realizados con objeto de examinar la respuesta de plantas silvestres a la aplicación de 9-HOT habían puesto de manifiesto que el fenotipo de waving radicular conlleva la formación de depósitos de calosa, la producción de ion superóxido, y la activación de la expresión de genes específicos de la planta, de los que un alto porcentaje corresponden a genes de respuesta a la infección de patógenos. Estos parámetros han sido utilizados con objeto de examinar la insensibilidad de los mutantes *noxy6* y *noxy22* a la aplicación de 9-HOT y los resultados obtenidos aparecen recogidos en las Figuras 7 y 8.

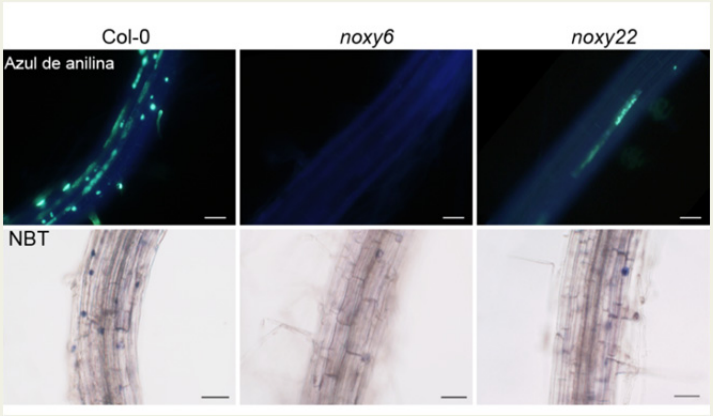


Figura 7. Caracterización fenotípica de los mutantes *noxy6* y *noxy22*.
Se muestran imágenes representativas de depósitos de calosa en raíces teñidas con azul de anilina y depósitos de azul tetrazolio revelados mediante tinción con NBT.

Las tinciones con azul de anilina y NBT, en raíces de plántulas crecidas en presencia de 9-HOT, pusieron de manifiesto un alto nivel de acúmulos de calosa y de ion superóxido en las plantas silvestres examinadas, y que ambas respuestas eran claramente más débiles en los dos mutantes *noxy6* y *noxy22* caracterizados.

De la misma manera, el análisis de la expresión de los genes de respuesta a 9-HOT *POX*, (pyridine nucleotide oxidoreductase, At5g22140), *ABC* (ABC transporter, At1g15520) y *FOX* (FAD-binding oxidoreductase, At1g26380) (Vellosillo y col., 2007) puso de manifiesto la inducción de su expresión en las raíces de plantas control a las tres horas de la aplicación de 9-HOT, mientras que los niveles de expresión de los genes examinados en los mutantes *noxy6* y

noxy22 fue, en todos los casos, inferior al correspondiente al de las plantas control.

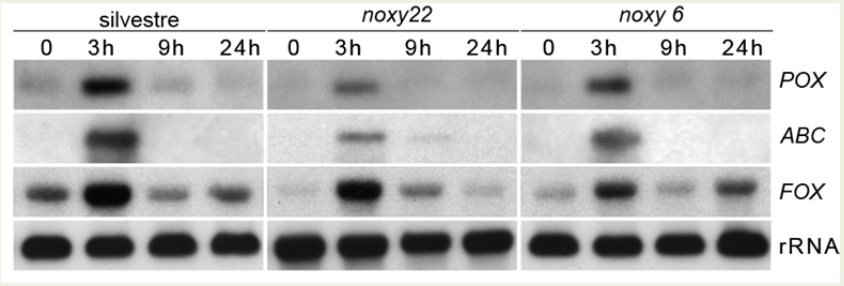


Figura 8. Caracterización molecular de la respuesta a 9-HOT de los mutantes *noxy6* y *noxy22.* Cinética de la acumulación de los transcritos de los genes de respuesta a 9-HOT en plántulas de 10 días de edad del tipo silvestre, *noxy22* y *noxy6* tratadas con una solución a 25 µM de 9-HOT. Los filtros conteniendo los ARNs obtenidos a distintos tiempos de tratamiento se hibridaron con sondas preparadas a partir de los genes *POX, ABC* y *FOX.*

Todos los resultados descritos confirmaban la insensibilidad de los mutantes *noxy6* y *noxy22* a la aplicación de 9-HOT y por lo tanto la participación de los genes *NOXY6* y *NOXY22* en la señalización de los procesos activados en respuesta al tratamiento con esta oxilipina.

2.3. CLONACIÓN Y CARACTERIZACIÓN DE LOS MUTANTES *NOXY6* Y *NOXY22*

Con objeto de determinar la dominancia o recesividad de las mutaciones *noxy6* y *noxy22*, ambos mutantes se cruzaron con plantas silvestres del ecotipo Columbia y se examinó la segregación de ambas mutaciones en las poblaciones de semillas F1 y F2, procedentes de dichos cruces. Estos análisis pusieron de manifiesto que las dos mutaciones caracterizadas eran monogénicas y recesivas. Posteriormente, y con objeto de generar las poblaciones recombinantes utilizadas para realizar el cartografiado posicional de los genes de interés, los mutantes *noxy6* y *noxy22* se cruzaron con plantas silvestres del ecotipo C24 y se recogieron las poblaciones F2 recombinantes.

El uso de marcadores moleculares polimórficos entre estos dos ecotipos, nos permitió situar la mutación *noxy6* en el cromosoma 5, dentro de un intervalo genómico flanqueado por los SSLPs, MOK16 y CIW18. Dicho fragmento genómico contenía el gen *CTR1* (CONSTITUTIVE TRIPLE RESPONSE 1) que codifica una Ralf-like proteina kinasa, que actúa como regulador negativo de la señalización de etileno (Kieber y col, 1993).

De la misma manera, se procedió a definir la posición de la mutación *noxy22* en el cromosoma 3, dentro de un fragmento de ADN flanqueado por los SSLPs, F24M12-TGF Y F28P10. Posteriormente, y mediante el uso de marcadores adicionales generados en base a la información de las bases de datos de marcadores, MASC, SNP y TAIR Polymorfism, la posición de la mutación *noxy22* fue acotada dentro de una región de 252 kilo bases en la que se encuentra el locus *ETO1 (ETHYLENE OVERPRODUCER),* que actúa como regulador negativo de la producción de etileno. Así, la proteína ETO1 interacciona e inhibe la actividad de la enzima ACS (1-aminocyclopropane-1-carboxylate synthase (ACC synthase)) implicada en la síntesis de etileno, e interacciona además, con la proteína CUL3 (Cullin-based ubiquitin ligase) del complejo ubiquitin ligasa, favoreciendo la degradación de la proteína ACS. De esta manera la proteína ETO actúa a dos niveles distintos, inhibiendo la actividad del enzima ACS y promoviendo su degradación, para regular la producción de etileno de forma negativa (Woeste y col., 1999; Christians y col., 2009).

Los resultados de estos análisis abrían la posibilidad de que las dos mutaciones *noxy6* y *noxy22* examinadas, estuvieran afectando a la señalización y a la producción de etileno, y que la respuesta de la planta a esta hormona estuviera activada de forma constitutiva en ambos mutantes. Para confirmar esta posibilidad se procedió a examinar la triple respuesta característica de plantas con sobreproducción o activación constitutiva de la señalización de etileno (Guzman y Ecker, 1990). En esta respuesta las plantas desarrollan tres alteraciones fenotípicas características, cuando se germinan en la oscuridad, que consisten en presentar un hipocotilo más corto y engrosado, en el que la

parte apical aparece formando un gancho muy pronunciado, y una raíz también más corta que la observada en plantas no mutantes.

Figura 9. Análisis de la triple respuesta en plántulas del tipo silvestre, *noxy6* y *noxy22* crecidas en oscuridad.
Imágenes representativas de plántulas del tipo silvestre, *noxy6* y *noxy22* crecidas en oscuridad durante 4 días.

Como se muestra en la Figura 9, los resultados de este análisis revelaron con claridad que mientras el genotipo silvestre presentaba el crecimiento esperado en plántulas crecidas en ausencia de luz, las alteraciones fenotípicas observadas en los mutantes *noxy6* y *noxy22* ponían de manifiesto la activación constitutiva de la señalización de etileno y permitían predecir que dichas mutaciones podrían estar localizadas en los *loci CTR1* y *ETO1*, respectivamente.

El análisis de las secuencias de ADN correspondientes a dichos *loci* en los mutantes *noxy6* y *noxy22* permitió confirmar estos resultados. Así, la secuencia del *locus CTR1* en el mutante *noxy6*, presentaba una mutación en el nucleótido 1349 que consistía en un cambio nucleótidico de citosina (C) a timina (T). Esta mutación provoca un cambio de leucina (Leu) a fenilalanina (Phe) en el aminoácido 610 correspondiente al dominio quinasa de la proteína. Estos resultados permitían demostrar que la mutación *noxy6* correspondía a un nuevo alelo del gen *CTR1*, distinto de los identificados hasta el momento, que fue designado *ctr1-15*, en el que la proteína CTR1 conserva un cierto nivel de actividad.

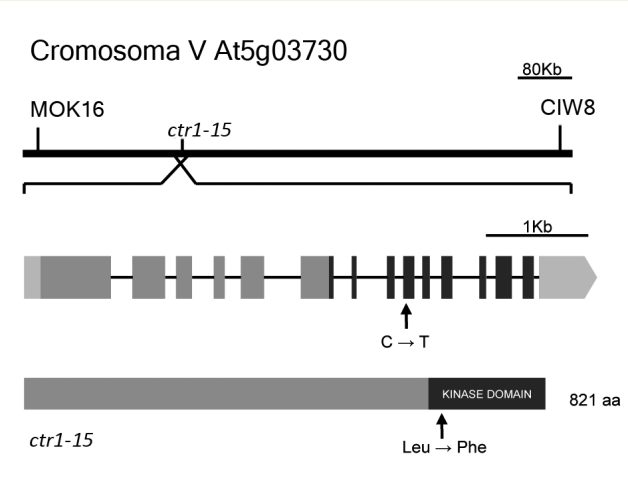


Figura 10. Cartografiado y representación esquemática de la secuencia del gen *CTR1* y de la proteína CTR1.
Esquema de la región del cromosoma 5 de *Arabidopsis* en la que se localiza el gen *CTR1*. En la representación genómica los exones están dibujados como cajas. La mutación *ctr1-15* provoca un cambio aminoacídico L_{610} F en el dominio quinasa señalado en el esquema de la proteína.

Al igual que en otros alelos previamente caracterizados, la longitud de la raíz en el mutante *ctr-15* era inferior a la correspondiente a las plantas silvestres, y presentaba numerosos pelos radiculares. Sin embargo el fenotipo del alelo *ctr1-15* no es tan severo como el de otros alelos tales como por ejemplo *ctr1-1,* en el que la falta de actividad catalítica provoca una importante alteración en el desarrollo de la planta. El hecho de que el mutante *ctr1-15* mostrara un fenotipo más suave que el mutante *ctr1-15*, indicaba que probablemente correspondía a un alelo hipomórfico

De la misma manera, el análisis de la secuencia correspondiente al gen *ETO1* en el mutante *noxy22* reveló un cambio nucleotídico de C a T en el nucleótido 821, que conlleva una sustitución de prolina (Pro) a leucina (Leu) en el amino acido 265 localizado en el dominio BTB de la proteína ETO1. Al igual que en otros alelos mutantes *eto1* previamente caracterizados, el alelo correspondiente a la mutación *noxy22*, designado *eto1-14,* presenta una reducción en la longitud de la raíz y un mayor número de pelos radiculares, aunque el hecho de que las alteraciones fenotípicas asociadas a esta mutación sean menos severas que las observadas en otros alelos sugiere que la proteína codificada en el mutante *eto1-14* conserva parte de su actividad.

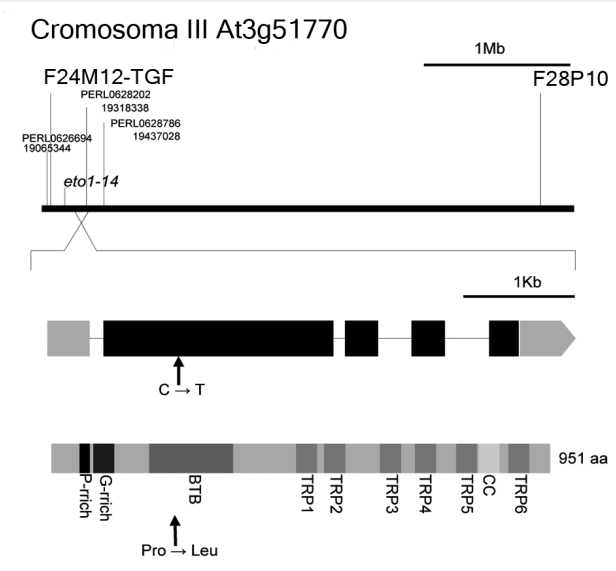


Figura 11. Cartografiado y representación esquemática de la secuencia del gen *ETO1* y de la proteína ETO1.

Esquema de la región del cromosoma 3 de *Arabidopsis* en la que se localiza el gen *ETO1*. En la representación genómica los exones están dibujados como cajas. La mutación *eto1-14* provoca un cambio aminoacídico P_{265} L en el dominio BTB señalado en el esquema de la proteína.

2.4. EL ET ACTÚA COMO REGULADOR NEGATIVO DE LA RESPUESTA AL 9-HOT

El hecho de que las mutaciones *ctr1-15* y *eto1-14*, seleccionadas en base a su insensibilidad a 9-HOT, provocaran la activación constitutiva de la respuesta a etileno, indicaba que la producción de esta hormona podría ejercer un efecto negativo en la activación de la ruta de señalización activada en respuesta a 9-HOT. En este caso, era posible predecir que la aplicación exógena de etileno podría inhibir, igualmente, el waving radicular producido en respuesta a 9-HOT. Para evaluar esta posibilidad se procedió a examinar el fenotipo radicular en plántulas silvestres crecidas en presencia del precursor de etileno ACC (1-aminocyclopropane-1-carboxylic acid) o de 9-HOT, en comparación con el fenotipo de plántulas crecidas simultáneamente en presencia de ambos compuestos. En estos ensayos, además del fenotipo de waving radicular, se examinaron otras respuestas a la aplicación de 9-HOT tales como la acumulación de depósitos de calosa y de ion superóxido. Los resultados de estos análisis (Figura12) permitieron comprobar que la aplicación de etileno inhibe las tres respuestas examinadas (el waving radicular, la formación de

depósitos de calosa y la generación de ion superóxido) y por tanto que el etileno inhibía la respuesta de la planta al 9-HOT.

Para examinar el efecto antagonista del etileno en más detalle se procedió a realizar el mismo tipo de análisis utilizando el mutante *ein2-5,* insensible a etileno, en el que la falta de funcionalidad de la proteína EIN2 impide la activación de los genes de respuesta a etileno. De esta manera, la utilización del mutante *ein2-5* permitía examinar si el efecto antagonista del etileno sobre la activación de la respuesta a 9-HOT, requiere o no de la activación de la señalización dirigida por la acción de esta hormona.

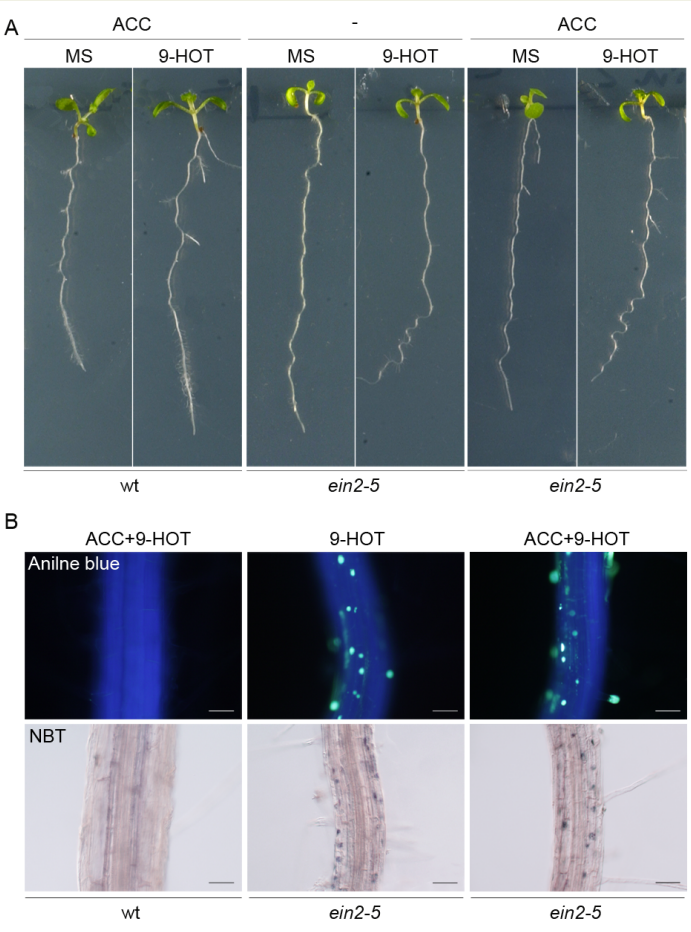


Figura 12. Caracterización fenotípica de plántulas silvestres y del mutante *ein2-5* en respuesta a ACC y 9-HOT.
(A) Imágenes representativas de plántulas de 3 días crecidas en medio MS y transferidas, para continuar su crecimiento durante 3 días más, a medios MS con 9-HOT (25 µM), ACC (2 µM) o 9-HOT (25 µM) en combinación con ACC (2 µM).
(B) Tinciones de las raíces en los tratamientos anteriormente descritos. Se muestran imágenes representativas de depósitos de calosa en raíces teñidas con azul de anilina y de la acumulación de iones superóxido revelados mediante tinción con NBT.

Los resultados de estos análisis revelaron que la aplicación de ACC no inhibía la respuesta del mutante *ein2-5* a la aplicación de 9-HOT, como se concluye del hecho de que las plantas *ein2-5* respondían de la misma manera al 9-HOT si se aplica solo, o en combinación con ACC. Así, el mutante *ein2-5* manifestaba un fenotipo de waving radicular con formación de calosa y generación de ion superóxido, en respuesta al tratamiento simultaneo con 9-HOT y ACC, lo que permite concluir que la inhibición ejercida por el etileno sobre la ruta de señalización del 9-HOT requiere de la acción del regulador EIN2. Adicionalmente, estos resultados demuestran que la actividad del 9-HOT no se ve afectada por la presencia de ACC en el medio de crecimiento.

El hecho de que la acción antagonista del etileno sobre la respuesta de la planta a 9-HOT fuera dependiente de la proteína EIN2, permitía pensar que el nivel de activación de los genes inducibles por 9-HOT podría ser mayor en las plantas *ein2-5* que en las plantas silvestres.

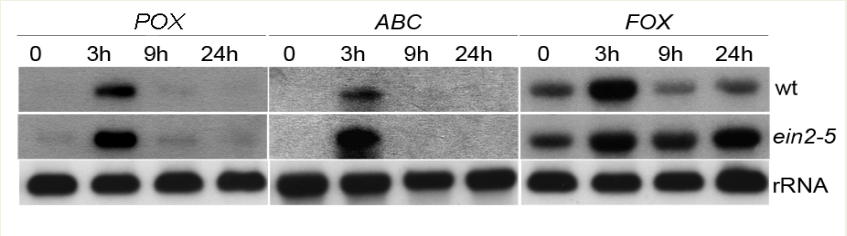


Figura 13. Caracterización molecular de la respuesta a 9-HOT del mutante *ein2-5.*
Cinética de la acumulación de los transcritos de los genes de respuesta a 9-HOT en plántulas de 10 días de edad del tipo silvestre y de *ein2-5* cubiertas con una solución a 25 µM de 9-HOT. Los filtros conteniendo los ARNs obtenidos a distintos tiempos de tratamiento se hibridaron con sondas preparadas a partir de los genes *POX, ABC* y *FOX.*

Los estudios realizados con objeto de examinar esta posibilidad pusieron de manifiesto que, efectivamente, la expresión de los genes *POX, ABC* y *FOX*, inducibles en respuesta a 9-HOT, alcanzaba mayores niveles de inducción en los mutantes *ein2-5* que en las plantas silvestres, lo que permite concluir que la falta de activación de la señalización de etileno favorece la inducción de los genes de respuesta a 9-HOT.

3. EL 9-HOT INTERFIERE CON LA ACTIVACIÓN DE LA RUTA DE ETILENO

Debido a que el aumento de los niveles de etileno disminuye la respuesta de la planta a 9-HOT, se quiso examinar si de forma recíproca, el aumento de los niveles de 9-HOT ejercía un efecto antagónico en la activación de la respuesta de las plantas a la producción de etileno. En apoyo de esta posibilidad, los resultados mostrados en la Figura 12, indicaban que el acortamiento en la longitud de la raíz producido en respuesta a la presencia de ACC y, por tanto, de la activación de la ruta del etileno, experimentaba una reversión parcial cuando se añadía 9-HOT, conjuntamente con ACC, al medio de crecimiento.

3.1. ANÁLISIS MOLECULAR DE LA INTERACCIÓN 9-HOT-ACC

Para examinar el efecto inhibidor del 9-HOT en la activación de la respuesta a etileno se utilizaron plantas transgénicas portadoras de la construcción *35S:EIN3:GFP,* con expresión constitutiva del factor de transcripción EIN3. La estabilización de la proteína EIN3, en respuesta a la producción de etileno, provoca la activación de la expresión de los genes de respuesta a esta hormona y es, por tanto, clave en la señalización de esta ruta de transducción (Gua y Ecker, 2003).

Para examinar la interacción entre las rutas de señalización activadas en respuesta a 9-HOT y a etileno, se procedió a utilizar anticuerpos anti-EIN3 para analizar, mediante western blot, el nivel de proteína EIN3 en extractos obtenidos a partir de plántulas *35S:EIN3:GFP* tratadas solo con ACC, o con ACC y 9-HOT simultáneamente. De acuerdo a los resultados esperados, la presencia de ACC inducía una rápida acumulación en los niveles de proteína EIN3, que se observaba a los 45 min de su aplicación y alcanza su máximo nivel a los 90 min de tratamiento. La aplicación de 9-HOT junto con el ACC, provocó una importante disminución en los niveles de proteína EIN3 detectados, en comparación al nivel observado en las plántulas tratadas con ACC, apoyando el

efecto antagonista de las rutas de señalización reguladas por la acción del 9-HOT y del etileno (Figura 14A). En estos estudios, y con objeto de examinar la interacción del etileno con otras oxilipinas de interés, se procedió a analizar el efecto de las oxilipinas 9-KOT, 9-oxo-C_9 y JA en el nivel de acumulación de la proteína EIN3 que se induce en respuesta a ACC. En este punto cabe recordar que las tres oxilipinas examinadas inducen distintas respuestas en la planta y que se activan a través de rutas de señalización independientes entre si. Así, al igual que el 9-HOT, la aplicación de 9-KOT induce la formación de waving radicular, el 9-oxo-C_9 provoca la inhibición del crecimiento radicular con pérdida de la dominancia apical, y el JA provoca una inhibición generalizada del crecimiento radicular.

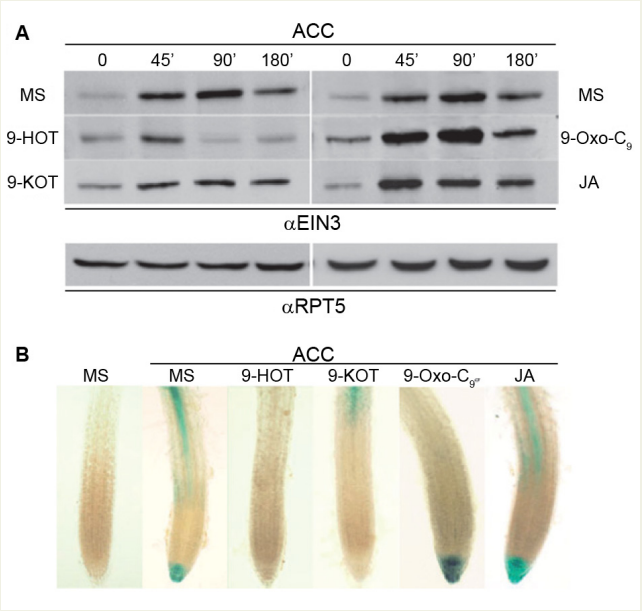


Figura 14 Caracterización de la señalización de ET a la aplicación de 9-HOT exógeno.
(A) Análisis de la acumulación de la proteína EIN3 en plántulas *35S::EIN3:GFP* de 6 días de edad, crecidas en medio MS y transferidas a medio MS con sólo ACC (2μM) o en combinación con 9-HOT, 9-KOT, JA o 9-Oxo-C9 (25 μM). Las proteínas fueron extraídas y examinadas a los tiempos indicados. Las membranas conteniendo las proteínas extraídas fueron incubadas con el anticuerpo anti-EIN3. El anticuerpo anti-RPT5 se usó como control de carga. (B) Ensayo de la actividad GUS en plantas transgénicas que contienen la construcción *EBS::GUS*. El ensayo GUS se realizó a las 24h tras el tratamiento con sólo ACC o con ACC en combinación con las oxilipinas 9-HOT, 9-KOT, JA o 9-Oxo-C9 (25 μM).

Los resultados de estos análisis mostraron que al igual que el 9-HOT, la aplicación de 9-KOT provocaba una disminución en el nivel de acumulación de la proteína EIN3 que se induce tras el tratamiento con ACC, mientras que, por el contrario, las dos oxilipinas restantes, 9-oxo-C_9 y JA, potenciaban la acumulación de EIN3, que alcanzó niveles más altos que los observados en la respuesta al tratamiento con ACC (Figura 14A). Los resultados obtenidos ponen de manifiesto la interacción de las oxilipinas en la ruta de señalización regulada por la acción del etileno, sobre la que pueden ejercer un efecto antagonista como en el caso del 9-HOT y del 9-KOT, o agonista como se observa en respuesta al tratamiento con las oxilipinas 9-oxo-C_9 y JA.

Los resultados obtenidos mediante la caracterización de las plantas *35S:EIN3:GFP,* fueron corroborados en ensayos realizados en paralelo utilizando la segunda línea transgénica *EBS::GUS*, en la que la expresión del gen marcador *GUS* se encuentra bajo el control del promotor sintético EBS (del inglés *ethylene binding site*) de respuesta a etileno y cuya expresión se activa como consecuencia de la estabilización de la proteína EIN3. En este caso, y de acuerdo a los resultados descritos anteriormente, se pudo observar que la aplicación de ACC indujo la expresión del gen GUS en el ápice de la raíz, y que dicha inducción disminuyó, de forma significativa, cuando el ACC se aplicaba simultáneamente con las oxilipinas 9-HOT o 9-KOT, inductoras del waving radicular. Por el contrario, el nivel de actividad GUS detectado aumentaba con respecto al observado en la respuesta a ACC, cuando las plantas se trataban conjuntamente con ACC y 9-oxo-C_9 o JA (Figura 14B).

Los resultados obtenidos ponen de manifiesto la interacción de las oxilipinas en la ruta de señalización regulada por la acción del etileno, sobre la que pueden ejercer un efecto antagonista, como en el caso del 9-HOT y del 9-KOT, o agonista como se observa en respuesta al tratamiento con las oxilipinas 9-oxo-C_9 y JA.

4. CARACTERIZACIÓN DE LA RESPUESTA A PATÓGENOS HEMIBIOTROFOS DEL MUTANTE *ETO1-14*

Los resultados descritos hasta el momento ponen de manifiesto la interacción de las rutas de señalización inducidas en respuesta a 9-HOT y etileno, en donde ambas señales ejercen un efecto antagonista recíproco. En base a esta circunstancia, y dado que la señalización activada por 9-HOT ejerce un papel positivo en la respuesta de defensa de la planta frente a la infección de bacterias hemibiotrofas, era posible predecir que el potencial defensivo de los mutantes con expresión constitutiva de etileno sería menor que el correspondiente al de las plantas silvestres. Para evaluar esta posibilidad se procedió a examinar la respuesta del mutante *eto1-14* a la infección de las cepas virulenta y avirulenta de la bacteria *Pst* DC3000 utilizadas en este estudio. Este ensayo se realizó únicamente en el mutante *eto1-14*, puesto que a diferencia del mutante *ctr1-15* presenta un porte similar al ecotipo silvestre que facilita los ensayos de inoculación realizados en este estudio.

4.1. CRECIMIENTO BACTERIANO, CARACTERIZACIÓN MOLECULAR Y SINTOMÁTICA EN RESPUESTA A BACTERIAS HEMIBIOTROFAS.

Para analizar la respuesta de la planta a la infección de las bacterias descritas se examinaron tres parámetros distintos: la tasa de crecimiento bacteriano, el nivel de activación de los genes de defensa, y la formación de los síntomas producidos en los tejidos infectados.

El recuento del número de bacterias a los tres días de la inoculación de las dos cepas examinadas, permitió observar un claro aumento en la tasa de crecimiento alcanzado en el mutante *eto1-14*, en relación al crecimiento correspondiente a las plantas control. Estas diferencias afectaban a las dos bacterias examinadas (Figura 15).

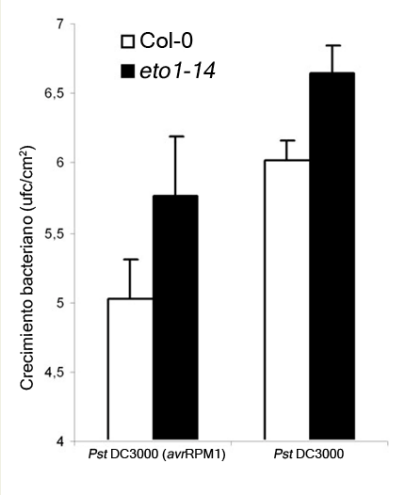


Figura 15. Análisis de plantas del tipo silvestre y del mutante *eto1-14* en infecciones bacterianas.
Nivel de crecimiento alcanzado por la cepa *Pst* DC3000 *avr*RPM1 (10^5 ufc mL^{-1}) y de *Pst* DC3000 (10^5 ufc mL^{-1}) en plantas del tipo silvestre y del mutante *eto1-14* a los 4 días de infección. Los datos corresponden a la media y errores estándar de 3 experimentos independientes.

El aumento de la susceptibilidad del mutante *eto1-14* estaba acompañado además, de una reducción significativa en el nivel de inducción de los genes de defensa caracterizados que era patente en las dos interacciones examinadas (Figura 16).

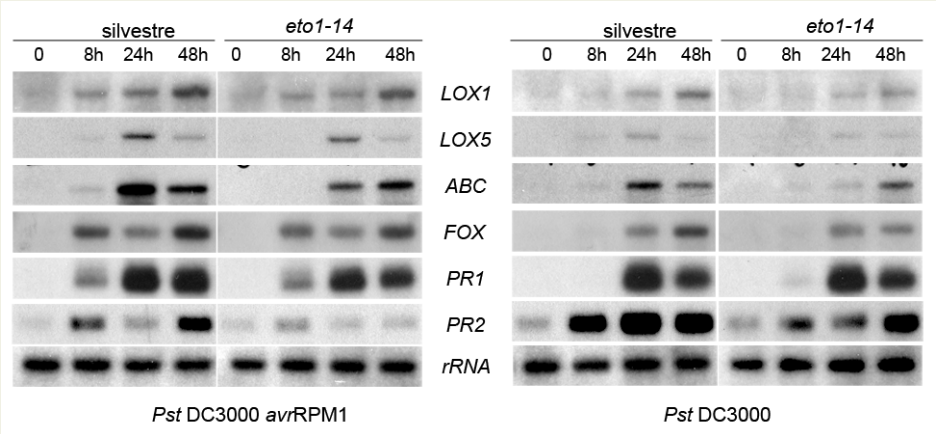


Figura 16. Caracterización molecular del mutante *eto1-14* en respuesta a la infección con bacterias.
Cinética de la acumulación de transcritos de genes de respuesta a 9-HOT y genes marcadores de respuesta a patógenos hemibiotrofos. La expresión de los genes se analizó en hojas de plantas de 4 semanas de edad del tipo silvestres y del mutante *eto1-14* inoculadas con las cepas *Pst* DC3000 *avr*Rpm1 (10^6 ufc/mL) y *Pst* DC3000 (10^6 ufc/mL). Los filtros conteniendo los ARNs obtenidos a distintos tiempos de infección se hibridaron con sonda preparadas a partir de los genes *LOX1, LOX5, ABC, POX, PR1* y *PR2*.

Al igual que en los dos parámetros descritos, el análisis de los síntomas de infección producidos en el mutante *eto1-14* reveló variaciones importantes con respecto a los observados en las plantas control.

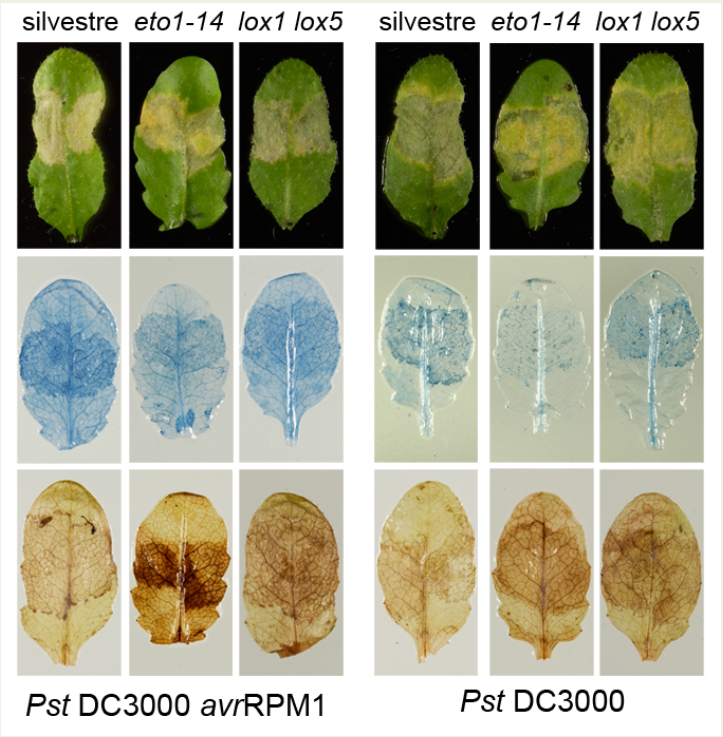


Figura 17. Análisis fenotípico de la respuesta a patógenos en plantas silvestres, y en los mutantes *eto1-14* y *lox1 lox5*.
Ejemplos representativos de las lesiones desarrolladas en plantas silvestres, *eto1-14* y *lox1 lox5* tras la inoculación de las dos cepas bacterianas examinadas.
Las imágenes de los síntomas, las tinciones con azul de tripano (azul) y las de diaminobencidina (marrón) se realizaron a las 48 h tras la inoculación bacteriana.

Como se observa en la Figura 17, mientras que en las plantas silvestres las lesiones producidas en respuesta a la inoculación con la cepa avirulenta, presentaban un aspecto necrótico característico de una respuesta hipersensible de defensa, dichas lesiones aparecían con una coloración clorótica en el mutante *eto1-14*. Esta característica se apreciaba igualmente en los síntomas formados frente a la cepa virulenta en los que la formación de áreas cloróticas era mayor en el mutante *eto1-14* que en las plantas control. Las lesiones descritas se examinaron en más detalle mediante tinción con azul de tripano (TPB), que tiñe de color azul las células muertas, y con diaminobenzidina (DAB) que tiñe de color marrón la presencia de H_2O_2. En este estudio se procedió a

teñir igualmente las lesiones producidas en el mutante *lox1 lox5* en el que a simple vista no se apreciaban diferencias acusadas en las lesiones formadas con respecto a las correspondientes a las plantas control.

Los resultados de estos análisis aparecen mostrados en la Figura 17 en la que se puede apreciar que la formación de clorosis muestra una relación inversa con la intensidad de la muerte celular, que es claramente menor en el mutante *eto1-14* que en las plantas control. Por el contrario existe una relación directa entre la formación de clorosis y la acumulación de H_2O_2 que es mayor en el mutante *eto1-14* que en las plantas silvestres. Al igual que en el mutante *eto1-14*, el análisis de las plantas *lox1 lox5* muestra una reducción en la intensidad de la muerte celular y un aumento en los niveles de H_2O_2 aunque las diferencias con las plantas control son menos acusadas que en el caso del mutante *eto1-14.*

5. RESPUESTA DE LOS MUTANTES *ETO1-14* Y *LOX1 LOX5* A LA GENERACIÓN DE ROS

La producción de ROS es una parte activa de la respuesta de defensa de la planta, en donde, como consecuencia del denominado estallido oxidativo, el oxígeno molecular puede convertirse a ion superóxido (O_2^-) y a peróxido de hidrógeno (H_2O_2), mediante una reacción de transferencia de electrones, o a oxígeno singlete ($^1O_2$), mediante un proceso de transferencia de energía (Apel y Hirt, 2004). La importancia de estos compuestos en la defensa vegetal se basa en los resultados que demuestran su actuación como señales celulares para inducir la expresión de genes específicos de la planta, así como su contribución a la creación del ambiente redox requerido para la activación de determinados procesos de señalización (Torres y col., 2006; Gechev y col., 2006; Yoshioka y col., 2009; O'Brien y col., 2012).

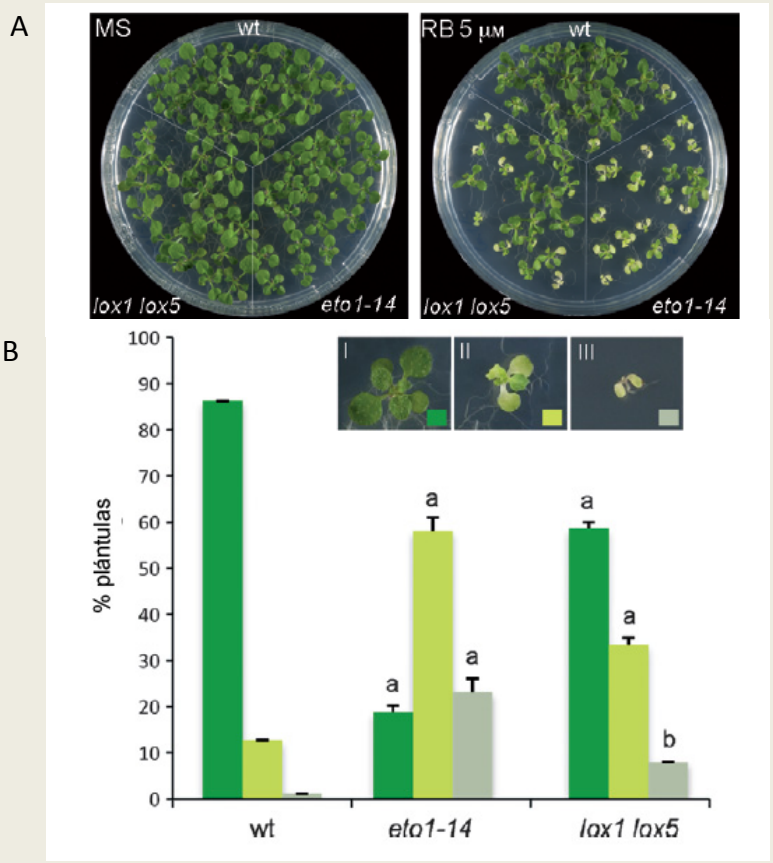


Figura 18. Análisis de las alteraciones fenotípicas en plantas silvestres, *eto1-14* y *lox1 lox5* crecidas en medio con RB.

(A) Fenotipo de la plántulas crecidas durante 15 días en medio MS (derecha) y medio MS con RB (5µM) izquierda. (B) Las alteraciones fenotípicas fueron cuantificadas en una escala (I, II y III) atendiendo a la severidad de los síntomas. Los valores representados corresponden a las medias y los errores estándares de tres experimentos independientes.

Las letras encima de las barras indican diferencias estadísticamente significativas entre los correspondientes mutantes y el tipo silvestre (test t-Student's t: $^{a}P < 0.001$, $^{b}0.001 < P < 0.01$).

La relevancia del estallido oxidativo en la defensa vegetal, junto con los resultados descritos en el apartado anterior, que mostraban la acumulación de H_2O_2 en las zonas infectadas de los mutantes *lox1 lox5* y *eto1-14*, sugería que estas mutaciones podrían interferir con los mecanismos implicados en la producción y/o en señalización de ROS. Siguiendo este razonamiento, era

posible prever que la aplicación exógena de compuestos generadores de ROS podría poner de manifiesto diferencias significativas en la respuesta de los mutantes *lox1 lox5* y *eto1-14* y de las plantas control a la presencia de dichos compuestos. Para evaluar esta posibilidad se procedió a crecer las plantas de interés en placas de medio MS conteniendo distintas concentraciones de H_2O_2, metilviologeno (generador de ion superóxido) y rosa de bengala (generador de oxígeno singlete), en las que se observaron las alteraciones fenotípicas producidas en respuesta a la presencia de los compuestos examinados.

Los resultados de estos análisis no permitieron observar diferencias significativas en la respuesta de los genotipos examinados, a la generación de H_2O_2 y de ion superóxido, así pues los síntomas asociados a la presencia de dichos compuestos mostraban la misma intensidad en todos ellos (resultados no mostrados). Por el contrario, la presencia de Rosa de Bengala (RB) provocaba importantes diferencias entre las plantas mutantes y las plantas control que era claramente visible en plántulas crecidas durante 15 días en medio MS conteniendo RB a la concentración de 5 µM. En estas condiciones las plántulas mutantes mostraban un aspecto clorótico y un tamaño menor al observado en las plantas control. La cuantificación de los síntomas en una escala de tres niveles I, II y III, de acuerdo a su severidad, revelaba un número mayor de plántulas del tipo III en los mutantes *eto1-14* y *lox1 lox5* y ponía de manifiesto el aumento de susceptibilidad de dichos mutantes al producto examinado, y en particular en el mutante *eto1-14*, en el que más del 80% de las plantas presentaban síntomas cloróticos (Figura 18).

5.1. RELACIÓN EN LA RESPUESTA AL $^1O_2$ Y EL DAÑO CELULAR

Con objeto de examinar el nivel de sensibilidad de los mutantes *lox1 lox5* y *eto1-14* al oxígeno singlete, se propuso determinar el nivel de daño celular producido por dicho compuesto. Para ello, se procedió a cuantificar el nivel de conductividad en hojas de plantas adultas infiltradas con una solución de rosa de bengala.

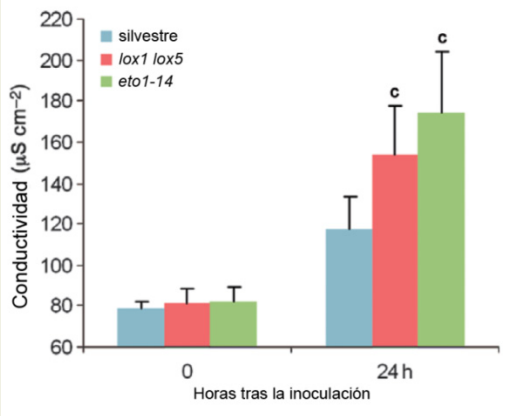


Figura 19 Medida de la conductividad en hojas de plantas silvestres, *lox1 lox5* y *eto1-14* infiltradas con rosa de bengala.
Cuantificación de los niveles de conductividad de plantas silvestres, *lox1 lox5* y *eto1-14* a tiempos 0 y 24h tras la infiltración con RB. Los datos corresponden a la media y los errores estándar de tres experimentos independientes. Las letras situadas encima de las barras indican diferencias significativas entre los mutantes correspondientes y las plantas silvestres (test t-Studend's $0,01 < P < 0,05$).

Los resultados de estos análisis permiten comprobar el aumento de la conductividad en los dos mutantes examinados, en relación a los valores determinados en las plantas tipo silvestre, y confirmar que el daño celular es mayor en el mutante *eto1-14* que aparece de nuevo como el más susceptible a la presencia del oxígeno singlete.

5.2. ANÁLISIS TRANSCRIPTÓMICO DE LA RESPUESTA A RB

Para profundizar en la caracterización de los mutantes *lox1 lox5* y *eto1-14*, se propuso examinar los cambios transcriptómicos producidos en respuesta al tratamiento con rosa de bengala, en comparación a los que ocurren en las plantas silvestres sometidas al mismo tratamiento. A este objeto se obtuvieron muestras de ARN a partir de plántulas crecidas durante 12 días en placas de MS y tratadas durante 3 h con una solución de rosa de bengala a la concentración de 10 µM. En todos los casos se obtuvieron cuatro réplicas independientes que se hibridaron en tres experimentos distintos, utilizando las micromatrices de Agilent 4 x 44K. Las hibridaciones realizadas permitieron llevar a cabo tres comparaciones distintas: i) el perfil transcriptómico de plantas silvestres tratadas con medio MS frente al de plantas tratadas con rosa de bengala, ii) el perfil transcriptómico de plantas silvestres tratadas con rosa de bengala frente al de

las plantas *lox1 lox5* sometidas al mismo tratamiento y iii) el perfil transcriptómico de plantas silvestres tratadas con rosa de bengala frente al del mutante *eto1-14* sometido al mismo tratamiento.

Los resultados de estos análisis se muestran en la Tabla S1 (incluida como material suplementario en el CD adjunto), en los que se reflejan los cambios de expresión observados en las hibridaciones realizadas.

El análisis de los cambios transcripcionales en plantas silvestres puso de manifiesto que el tratamiento con rosa de bengala afectaba a la expresión de un gran número de genes, entre los que 1262 aumentaban su nivel de expresión, mientras que la expresión de 570 genes disminuía como consecuencia del tratamiento.

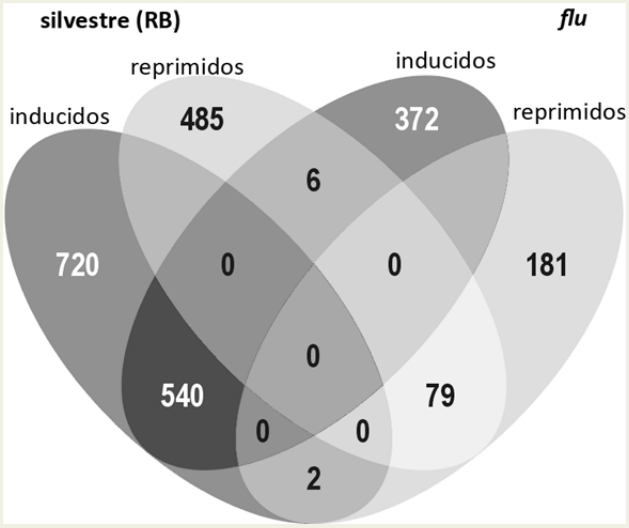


Figura 20. Comparación de los perfiles transcripcionales de plantas silvestres tratadas con RB y mutante *flu*.
Diagrama de Venn donde se muestra los conjuntos de genes inducidos y reprimidos en plantas silvestres, tras el tratamiento con RB y genes inducidos y reprimidos en el mutante *flu,* tras la generación del oxígeno singlete.

La comparación de estos resultados con el perfil transcriptómico caracterizado en el mutante *flu*, en el que la producción de oxígeno singlete aumenta en respuesta a un cambio en las condiciones de iluminación de oscuridad a luz, puso de manifiesto que el 60% de los genes que se inducen en el mutante *flu*, aumentan también su expresión en respuesta al tratamiento con rosa de bengala. Además el análisis de nuestros resultados permitió identificar la activación de genes de respuesta a oxígeno singlete, cuya expresión se induce

de forma específica frente a este tipo de ROS. Estos resultados ponían de manifiesto la validez de nuestros análisis e indicaban que en el tratamiento realizado el oxígeno singlete generado estaba actuando como molécula señalizadora.

La función biológica de los genes de respuesta a oxígeno singlete se examinó mediante el programa Gene Ontology (GO) en el que los genes de interés se agrupan de acuerdo a su correspondencia con determinadas funciones biológicas, denominadas términos (Tabla S del material suplementario en el CD adjunto). Dentro del grupo de genes inducibles por oxígeno singlete, los resultados de estos análisis revelaban una representación significativa de términos relacionados con la respuesta de la planta a distintos tipos de estrés, con la regulación de la expresión génica, el metabolismo de oxilipinas y la respuesta a etileno. Mientras que los términos relacionados con la modificación de la pared celular y el metabolismo de lípidos, aparecen sobre-representados dentro del grupo de genes cuya expresión disminuye en respuesta al oxígeno singlete.

La funcionalidad de los genes de respuesta a oxígeno singlete se examinó, igualmente, mediante su comparación con otros perfiles transcriptómicos, disponibles en las bases de datos utilizando la aplicación *Expression browser* disponible en la web de herramientas informáticas de *The Bio-Array Resource for Plant Biology.*

En estos análisis se pudo comprobar que una parte importante de los genes inducibles por oxígeno singlete corresponden a genes inducibles en respuesta a patógenos tales como *Pst* DC3000 (57%), *Pst* DC3000 *avr*RPM1 (54%) y *P. infestans* (58%). Igualmente, la respuesta a oxígeno singlete comparte un alto nivel de correspondencia con las respuestas a elicitores de naturaleza biótica tales como la flagelina (35%) y la proteína HrpZ (54%). Por otro lado cabe resaltar un alto nivel de correspondencia con la respuesta de las plantas a distintos tipos de estrés abiótico tales como el estrés salino (71%), el estrés osmótico (55%), el tratamiento con luz ultravioleta (55%), el estrés

oxidativo (48%) y el frio (43%). Finalmente, cabe mencionar la elevada coincidencia encontrada con genes inducidos en respuesta al tratamiento con el inhibidor de la síntesis de proteínas, cicloheximida (65%), y al inhibidor de la acción del etileno, el nitrato de plata (67%) (Figura 21).

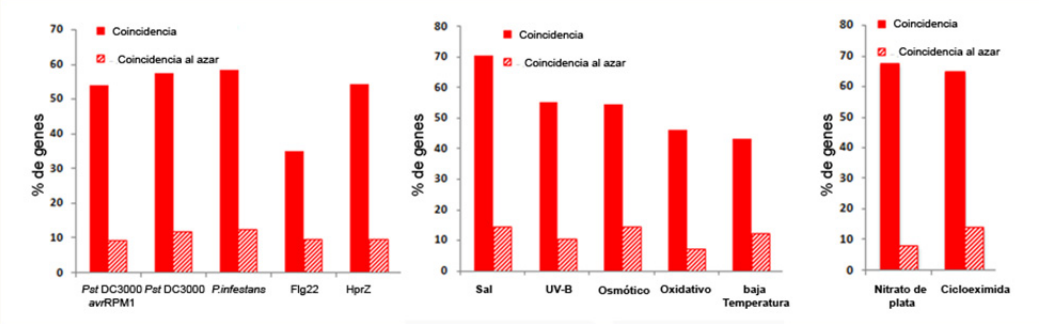


Figura 21. Análisis de los genes inducidos en el tratamiento con RB en plantas silvestres.
Comparación de los genes inducidos en plantas silvestres tras el tratamiento con RB y en respuesta a estreses bióticos (izquierda), abióticos (centro) y otros tratamientos (derecha).
Todos los estímulos mostrados presentaron diferencias significativas entre los genes que responden a RB y un grupo de 5000 genes seleccionados al azar, con los análisis transcripcionales disponibles en las bases de datos, usando un test binomial (P< 0,001).
Las barras rojas indican la correspondencia con genes inducidos para cada estímulo específico. Se usó un grupo de 5000 genes seleccionados al azar (barras rayadas) para validar la significación de estos análisis.

La validez de estos análisis se sustentó por los resultados obtenidos mediante la comparación de un grupo de 5000 genes, seleccionados al azar, con las mismas bases de datos que las utilizadas para examinar los genes de respuesta a oxígeno singlete, y en la que se comprobó, que la correspondencia de estos genes, con los genes de respuesta a los estímulos analizados, no superaba, en ninguno de los casos, el 13%.

El análisis de los cambios transcriptómicos que ocurren en los mutantes *lox1 lox5* y *eto1-14*, en respuesta al tratamiento con oxígeno singlete, puso de manifiesto la contribución de ambas mutaciones a la respuesta examinada (Figura 22).

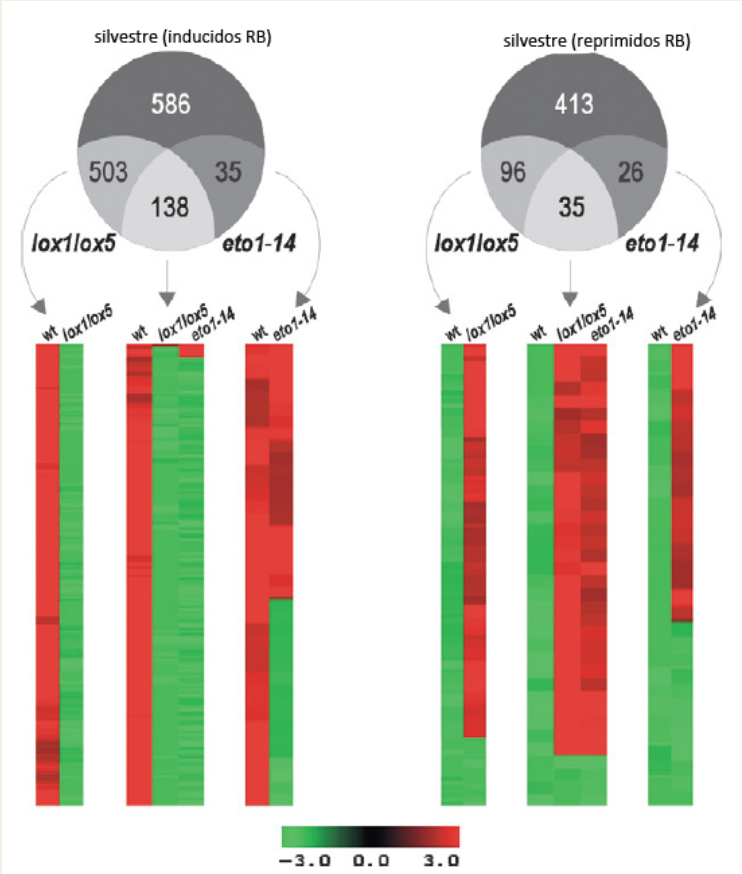


Figura 22. Análisis mediante micromatriz de genes de respuesta a RB en plantas silvestres, *lox1 lox5* y *eto1-14.*

Las muestras de ARNs fueron preparadas a partir de plantas de 12 días de edad tras 3 h de tratamiento con RB (10 μM). Se analizaron los genes inducidos y reprimidos con cambios en los niveles de expresión de dos o más veces.

Diagrama de Venn y ordenación jerárquica de los genes inducidos (izquierda) y reprimidos (derecha) en plantas silvestres tras el tratamiento con RB, así como de los genes alterados en plantas mutantes *lox1 lox5* y *eto1-14* tratadas con RB. Se muestran tres subgrupos que representan los genes cuya expresión varía en plantas silvestres y *lox1 lox5* (izquierda), en plantas silvestres y *eto1-14* (derecha), o en *lox1 lox5* y *eto1-14* simultáneamente (medio). Los colores indican los valores de los genes expresados diferencialmente en una escala de 3,0 a -3,0. Una lista completa de los genes se muestra en la Tabla S del CD adjunto.

Así en este estudio se pudo comprobar que la expresión de un alto porcentaje de los genes inducibles por oxígeno singlete en plantas silvestres, alcanzaba valores de activación menores (al menos dos veces) en los mutantes examinados (51% en *lox1 lox5* y 20% en *eto1-14*). Una situación similar ocurría con los genes reprimidos por oxígeno singlete, para los que la reducción de la expresión de un número significativo de genes (20% en *lox1 lox5* y 9% en *eto1-14*) era al menos dos veces menor en los mutantes caracterizados. Como dato destacable de estos análisis cabe mencionar, que la expresión del 9% de los genes de respuesta a oxígeno singlete (inducidos y reprimidos) varía simultáneamente en los mutantes *lox1 lox5* y *eto1-14*, y que el 74% de los genes cuya expresión varía en el mutante *eto1-14* con respecto a las plantas silvestres, se ven igualmente alterados en el mutante *lox1 lox5*, indicando que el ET afecta a una parte significativa de la respuesta transcripcional mediada por la actividad 9-LOX.

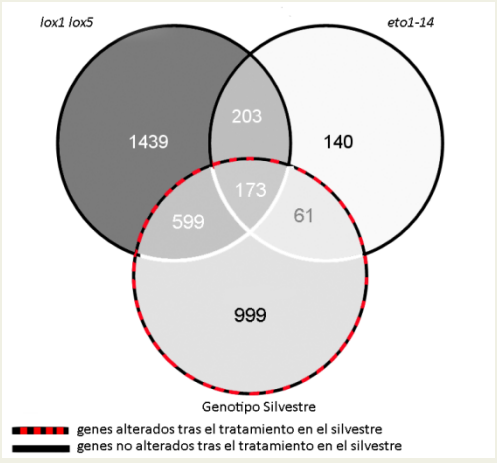


Figura 23. Análisis mediante micromatriz de genes de respuesta a RB en plantas silvestres, *lox1 lox5* y *eto1-14*.
Las muestras de ARNs fueron preparadas a partir de plantas de 12 días de edad tras 3 h de tratamiento con RB (10 μM). Se analizaron los genes inducidos y reprimidos con cambios en los niveles de expresión de dos o más veces.
El diagrama de Venn muestra los genes alterados en cada uno de los tres genotipos analizados y las coincidencias que presentan los grupos, silvestre, *lox1 lox5* y *eto1-14*.

Finalmente, la comparación de los cambios transcriptómicos ocurridos en plantas silvestres y mutantes, pone de manifiesto la existencia de un grupo importante de genes cuya expresión cambia exclusivamente en los mutantes *lox1 lox5* y *eto1-14*. Así, como se observa en el diagrama de Venn mostrado en la Figura 23, existe un grupo de 140 genes cuya expresión cambia

exclusivamente en el mutante *eto1-14* y un grupo de 1439 genes cuya expresión varía en el mutante *lox1 lox5* pero no en los dos genotipos restantes (*eto1-14* y plantas silvestres). Además estos análisis ponen de manifiesto un tercer grupo de 203 genes cuya expresión cambia simultáneamente en los mutantes *lox1 lox5* y *eto1-14*, pero no en las plantas silvestres.

El estudio de la función biológica de estos genes revela el enriquecimiento en términos asociados con estrés abiótico y fotosíntesis, en el caso de los genes cuya expresión varía exclusivamente en el mutante *lox1 lox5*, y en términos relacionados con el metabolismo de aminoácidos, fenilpropanoides y flavonoides en el caso de los genes correspondientes al mutante *eto1-14.* En cuanto a los genes que cambian simultáneamente en ambos mutantes, pero no en plantas silvestres, cabe destacar el enriquecimiento en términos relacionados con la respuesta a estrés oxidativo.

5.3. ANÁLISIS MOLECULAR DE LA RESPUESTA A RB

El análisis de los genes inducibles por oxígeno singlete en las plantas examinadas, nos permitió comprobar que la expresión de dos de los tres genes utilizados como marcadores de la respuesta a 9-HOT (*FOX* y *ABC*) aumentaba en plantas silvestres tratadas con rosa de bengala, y que su nivel de inducción era menor en los dos mutantes examinados. Para validar estos resultados se realizaron ensayos de northern blot en los que se examinó la expresión de los dos genes descritos, así como de un tercer gen (*POX*) utilizado en nuestros estudios como marcador de la respuesta a 9-HOT. En estos ensayos, la expresión de los tres genes descritos se examinó tanto en plantas silvestres como en mutantes *lox1 lox5* y *eto1-14* sometidos a un tratamiento con rosa de bengala. Además, y dado el efecto antagónico del etileno en la señalización activada por 9-HOT, la expresión de los genes descritos se analizó en plantas mutantes *ein2-5* insensibles a etileno.

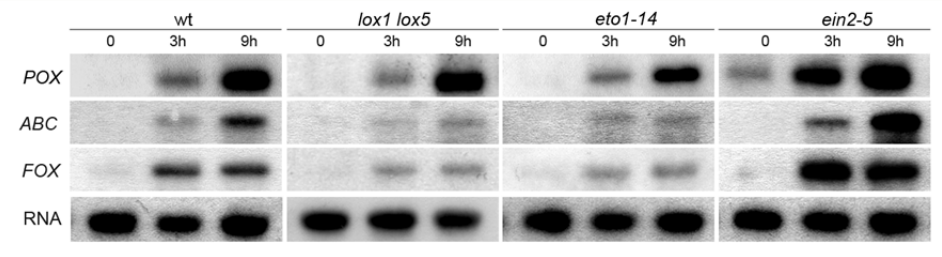


Figura 24. Caracterización molecular de la respuesta a RB en plantas silvestres y en los mutantes *lox1* lox5, *eto1-14* y *ein2-5.*

Cinética de la acumulación de los transcritos de los genes de respuesta a 9-HOT en plántulas de 10 días de edad del tipo silvestre, *lox1* lox5, *eto1-14* y *ein2-5* tratadas con una solución a 10 µM de RB. Los filtros conteniendo los ARNs obtenidos a distintos tiempos de tratamiento se hibridaron con sondas preparadas a partir de los genes *POX*, *ABC* y *FOX*.

Los resultados de estos análisis (Figura 24) permitieron observar que los tres genes examinados se inducían en plantas silvestres a las tres y nueve horas del tratamiento con rosa de bengala y que su nivel de inducción disminuía en los mutantes *lox1 lox5* y *eto1-14* con respecto al nivel detectado en las plantas control. Además, los resultados obtenidos en el mutante *ein2-5* revelaban un aumento en la expresión de los genes *POX*, *ABC* y *FOX* en respuesta al tratamiento con RB. Los resultados de estos análisis ponían de manifiesto el paralelismo entre las respuestas de la planta a la aplicación de 9-HOT y oxígeno singlete, así como el efecto antagónico del etileno en la activación de las respuestas a oxígeno singlete y 9-HOT.

DISCUSIÓN

DISCUSIÓN

Los estudios realizados con objeto de examinar la función de las enzimas 9-LOX, y de los derivados lipídicos generados a través de la ruta de síntesis de oxilipinas iniciada por la acción de estas enzimas, indicaban su participación en la defensa de las plantas frente a patógenos biotrofos. Además, los resultados de estos estudios mostraban la acción defensiva de dos de los derivados de está ruta de síntesis, en concreto del ácido 9-hidroxilinolénico (9-HOT) y el ácido 9-cetolinolénico (9-KOT). La producción de ambos compuestos induce la expresión de genes específicos de la planta, activa respuestas de defensa tales como la acumulación de depósitos de calosa y la generación de estrés oxidativo, y contribuye a la regulación de la homeostasis hormonal durante la respuesta de la

planta a la infección de bacterias virulentas.(Vellosillo y col., 2007; Vicente y col., 2011).

Nuestro interés en examinar en mayor detalle la función de esta ruta de síntesis de oxilipinas en la defensa vegetal, así como la acción de las oxilipinas producidas como consecuencia de su activación, nos condujo a generar y a examinar dos tipos de plantas mutantes deficientes, respectivamente, en la producción de oxilipinas y en su señalización. A este objeto, en este estudio generamos un doble mutante *lox1 lox5*, que contiene una inserción de ADN-T en cada uno de los dos genes 9-LOX (*LOX1* y *LOX5*) identificados en *Arabidopsis* y que carece por tanto de actividad 9-LOX (Figura 4). Además, y como mutantes de señalización, se procedió a la caracterización de los mutantes *noxy6* y *noxy22*, aislados en base a su insensibilidad a la respuesta de waving radicular inducida por 9-HOT en plantas de *Arabidopsis* silvestres. (Vellosillo y col., 2007).

El análisis de la respuesta del mutante *lox1 lox5* frente a la infección de las bacterias *Pst* DC3000 *avr*RPM1 (avirulenta) y *Pst* DC3000 (virulenta) puso de manifiesto, que la falta de actividad 9-LOX aumenta la susceptibilidad de la planta a la infección de bacterias patógenas, como se demuestra por el aumento en la tasa de crecimiento de la bacteria *Pst* DC3000 en el doble mutante *lox1 lox5*, en comparación al crecimiento alcanzado en las plantas silvestres (Figura 5A). A diferencia de estos resultados, el crecimiento de la bacteria avirulenta *Pst* DC3000 *avr*RPM1 no experimentaba diferencias significativas entre las plantas silvestres y el doble mutante examinado, lo que ponía de manifiesto el papel positivo de las enzimas 9-LOX en la defensa de la planta frente a la infección de bacterias virulentas. Al igual que en este trabajo, los resultados derivados de la caracterización del mutante *lox1* de *Arabidopsis* ponen de manifiesto el papel de las enzimas 9-LOX en la defensa frente a bacterias virulentas (Huwang and Huwang, 2010, Vicente y col., 2011). Sin embargo, cabe resaltar que, mientras que el crecimiento alcanzado por la bacteria *Pst* DC3000 en el mutante *lox1* es cinco veces superior que el de las plantas control (Hwang y Hwang, 2010; Vicente y col., 2011), la tasa de crecimiento alcanzada en el doble mutante *lox1*

lox5, carente de las dos actividades 9-LOX detectadas en *Arabidopsis*, es diez veces mayor que la de las plantas control, lo que demuestra la contribución de los dos genes 9-LOX, *LOX1* y *LOX5*, en la defensa de la planta.

La caracterización inicial de los mutantes *noxy6* y *noxy22*, permitió comprobar su insensibilidad a 9-HOT como se demuestra del hecho de que la activación de las respuestas a la aplicación de 9-HOT examinadas (waving radicular, producción de calosa, generación de ROS y activación de la expresión de genes inducibles por 9-HOT), era claramente inferior en los mutantes *noxy6* y *noxy22* que en las plantas control (Figuras 6, 7, y 8). Por otro lado, el hecho de que dichos mutantes respondían igual que las plantas control a las alteraciones fenotípicas producidas por oxilipinas tales como 9-oxo-C_9 y JA, inductoras de rutas de señalización independientes a la activada por 9-HOT, indicaban que la insensibilidad de los mutantes *noxy6* y *noxy22* afectaba primariamente a los procesos de señalización activados en respuesta a 9-HOT.

Al igual que con la respuesta al 9-HOT, el tratamiento de los mutantes *noxy6* y *noxy22* con otras oxilipinas inductoras de waving radicular, como por ejemplo el 9-KOT, reveló su insensibilidad frente a este tipo de oxilipinas (no mostrado), y puso de manifiesto que la identificación y caracterización de los mutantes *noxy6* y *noxy22*, contribuiría a la identificación de los procesos de señalización regulados en respuesta a la producción de 9-HOT, así como de otras oxilipinas inductoras del waving radicular.

El clonaje posicional de los mutantes *noxy6* y *noxy22* reveló su localización en los genes *CTR1* (CONSTITUTIVE ETHYLENE RESPONSE1) y *ETO1* (ETHYLENE OVERPRODUCER1), respectivamente. De acuerdo a la posible sobreproducción de etileno, los mutantes *noxy6* y *noxy22* muestran la triple respuesta característica de la activación de la ruta de señalización regulada por esta hormona (Figura 9), confirmando que correspondían a nuevos alelos de los genes *CTR1* y *ETO1*, que fueron renombrados, *ctr1-15* y *eto1-14,* respectivamente.

La falta de respuesta a la presencia de 9-HOT de los mutantes *ctr1-15* y *eto1-14* sugería que la producción de ET interfería con la señalización del 9-HOT (Figuras 6, 7 y 8). En apoyo a esta conclusión, se observa que la aplicación exógena del precursor de etileno, ACC, interfiere con la respuesta de las plantas a 9-HOT, como se concluye de los resultados que muestran que la presencia de ambos compuestos inhibe el waving radicular, la formación de depósitos de calosa y la generación de ROS característicos de la respuesta al 9-HOT. Igualmente, el hecho de que el mutante *ein2-5*, insensible a etileno, responde a la aplicación de ACC y 9-HOT induciendo una respuesta al 9-HOT (waving radicular, formación de depósitos de calosa y generación de ROS) similar a la de las plantas silvestres, y que la expresión de los genes de respuesta a 9-HOT alcanza niveles más altos en el mutante *ein2-5* que en las plantas silvestres (Figuras 12 y 13), confirman la acción antagonista del etileno en la vía de señalización del 9-HOT, y que dicha inhibición se establece a través de la ruta de señalización regulada por la acción del etileno.

Además del efecto inhibidor del ET en la señalización del 9-HOT, los resultados de este trabajo demuestran que el 9-HOT interfiere con la activación de la respuesta a etileno. Así, la presencia de 9-HOT en el medio de crecimiento disminuye el efecto del ACC en la reducción de la longitud de la raíz. Igualmente, la aplicación de 9-HOT interfiere con la acumulación de la proteína EIN3-GFP que se produce en respuesta al tratamiento con ACC en plantas transgénicas *35S::EIN3-GFP*, así como de la actividad GUS en las raíces de las plantas *EBS:GUS* (Figura 14). Este conjunto de resultados permite concluir que los dos compuestos examinados, el etileno y el 9-HOT ejercen un efecto antagonista recíproco y bidireccional.

El análisis del efecto de oxilipinas, distintas al 9-HOT en la ruta de señalización del etileno, permiten observar que el 9-KOT (inductor de waving radicular) ejerce un efecto antagonista, mientras que el JA o el 9-Oxo-C_9, actúan como agonistas favoreciendo la señalización del etileno. Así, mientras que el 9-KOT, interfiere con el nivel de acumulación de la proteína EIN3-GFP y la

actividad GUS que se induce en respuesta al tratamiento con el precursor de etileno ACC, en las plantas transgénicas *35S::EIN3-GFP* y *EBS::GUS*, la aplicación de 9-Oxo-C_9, y el JA potencian ambas respuestas. La interacción positiva entre las vías de señalización reguladas por la acción de las hormonas JA y ET ha sido descrita con anterioridad. Así, ambas hormonas actúan de forma positiva y coordinada durante la activación de la respuesta de defensa frente a la infección de patógenos necrotrofos (Penninckx y col., 1998; Lorenzo y col., 2003; Zhu y col., 2011; Zander y col., 2011). Además, los resultados de este trabajo revelan la interacción de otras oxilipinas con la ruta de señalización regulada por ET, en donde pueden ejercer un efecto agonista, como en el caso del 9-Oxo-C_9, o antagonista como la descrita para el 9-HOT y el 9-KOT. El hecho de que la acción del 9-HOT y el 9-KOT (derivados de la acción de las 9-LOX) en la señalización de ET, difiera de la caracterizada con el 9-Oxo-C_9 (sintetizada igualmente por la vía de las 9-LOX) y el JA (sintetizado a través de la ruta de las 13-LOX), apoya los resultados que ponen de manifiesto la especialización funcional de las oxilipinas y que la acción de las distintas oxilipinas varía de acuerdo a su estructura molecular, y no a la ruta bioquímica implicada en su producción.(Vellosillo y col., 2007)

Según discutimos anteriormente, los resultados derivados de la caracterización del mutante *lox1 lox5* demuestran la acción positiva de las enzimas 9-LOX en la activación de la respuesta de defensa frente a la infección de bacterias virulentas. Por otro lado, la identificación de los mutantes *ctr1-15* y *eto1-14* ha puesto de manifiesto que, la activación constitutiva de la señalización de etileno interfiere con la activación de la ruta de señalización que controla la respuesta a 9-HOT. De acuerdo a estos resultados el análisis de la respuesta a la infección de bacterias patógenas, reveló el aumento de la susceptibilidad del mutante *eto1-14* frente a las dos cepas bacterianas aquí examinadas, *Pst*DC3000 *avr*RPM1 (avirulenta) y *Pst*DC3000 (virulenta) (Figuras 15, 16 y 17). En estos ensayos, la formación de los síntomas asociados a la infección revela diferencias importantes con respecto a los síntomas que se forman en las

plantas control (Figura 17). Así, las lesiones formadas en las plantas *eto1-14*, tienen un aspecto clorótico, a diferencia del aspecto preferentemente necrótico que presentan las lesiones formadas en las plantas silvestres. Este efecto era patente a simple vista y se observaba en las hojas inoculadas con los dos tipos de bacterias, avirulentas y virulentas. El análisis detallado de estas lesiones, mediante la tinción con azul de tripano y DAB, puso de manifiesto que la formación de lesiones cloróticas estaba acompañada de un importante aumento en los niveles de H_2O_2. Aunque con menor intensidad que la observada en el mutante *eto1-14*, el análisis de las lesiones formadas en el mutante *lox1 lox5*, revela también un nivel de acumulación de H_2O_2 mayor al detectado en las plantas silvestres. Estos resultados ponen de manifiesto una alteración en los procesos de producción y regulación de ROS y sugieren que dicha alteración podría estar asociada al aumento de la susceptibilidad de los mutantes *lox1 lox5* y *eto1-14* frente a la infección de bacterias patógenas.

La participación de las enzimas 9-LOXs y del ET como reguladores de la producción y acumulación de ROS, se sustenta además a partir de los experimentos dirigidos a examinar la respuesta de ambos mutantes a la generación de ROS, en los que se observa el aumento de la susceptibilidad de los mutantes *lox1 lox5* y *eto1-14* a la producción de oxígeno singlete (Figura 18). La susceptibilidad de los mutantes *lox1 lox5* y *eto1-14* al oxígeno singlete se confirma con los resultados que demuestran un mayor nivel de daño celular en las hojas de los mutantes *lox1 lox5* y *eto1-14* que en las plantas silvestres en respuesta a la infiltración de oxígeno singlete (Figura 19). Estos dos grupos de resultados indican el defecto de los mutantes caracterizados en los mecanismos de producción y detoxificación de la planta frente a este tipo de estrés oxidativo. Por otro lado, el hecho de que un alto porcentaje de los 1832 genes que modifican su expresión en respuesta a la generación de oxígeno singlete, varía con respecto al nivel observado en la plantas silvestres en los mutantes *lox1 lox5* (51%) y *eto1-14* (12%), respectivamente, demuestra la participación de las enzimas 9-LOX y del ET en la regulación de la respuesta a este tipo especies

reactivas de oxígeno (Figuras 22 y 23). Así mismo, y dado que el nivel de activación y represión génica observado en ambos mutantes es inferior al detectado en las plantas silvestres, es posible predecir que el defecto asociado a estas mutaciones pueda disminuir la capacidad de las plantas para responder y regular la producción de oxígeno singlete y por tanto su hipersusceptibilidad frente a este tipo de compuestos.

Las investigaciones dirigidas a examinar el papel del ROS en la defensa vegetal han estado preferentemente dirigidas a estudiar la función del O_2^- y del H_2O_2. Los resultados obtenidos en este trabajo, revelan la acción directa o indirecta del oxígeno singlete ($^1O_2$) en la respuesta de la planta frente a la infección de patógenos. En apoyo de esta posibilidad cabe mencionar que una gran parte de los genes inducidos por oxígeno singlete corresponde a genes inducidos por patógenos tales como *Pst* DC3000 (57%), *Pst* DC3000 *avr*RPM1 (54%) y *P. infestans* (58%), así como por elicitores de naturaleza biótica tales como la flagelina (35%) y la proteína HrpZ (54%). Estos resultados, junto con el aumento de los niveles de H_2O_2 observados en los mutantes *lox1 lox5* y *eto1-14* durante la respuesta de la planta a la infección bacteriana, pone de manifiesto el papel de estas rutas de señalización implicadas en la regulación de estrés oxidativo.

El hecho de que las mutaciones *lox1 lox5* y *eto1-14* puedan afectar a distintos tipos de ROS ($^1O_2$ y H_2O_2) revela la participación de las oxilipinas producidas por la acción de las 9-LOXs y de la ruta del ET en rutas de señalización implicadas en la regulación de distintos tipos de ROS. Alternativamente, es posible que las alteraciones observadas se produzcan como consecuencia de la interacción entre las rutas de señalización que regulan la producción y acumulación de distintos tipos de ROS. En este sentido cabe mencionar los resultados publicados por Laloi y col. (2007) en los que se demuestra el efecto antagonista de las rutas de señalización que regulan la respuesta de la plantas a la producción de $^1O_2$ y H_2O_2. Con independencia de

estas dos posibilidades, los resultados descritos ponen de manifiesto la participación de las rutas caracterizadas (9-LOXs y ET) en el control del estrés oxidativo durante la respuesta de la planta a la infección por patógenos, en donde la producción y eliminación de las ROS es esencial para activar una respuesta de defensa. En este punto, es importante mencionar el efecto del 9-HOT como inductor de ROS (Figura 7). En este sentido cabe proponer que el 9-HOT, a través de la generación de ROS, podría contribuir a la activación de la expresión de los genes *9-LOX* para aumentar y amplificar su producción, y por tanto para inducir los genes implicados en la regulación y en el control de este proceso, permitiendo, así, la activación de una respuesta de defensa eficaz y controlada, necesaria para la supervivencia de las plantas.

CONCLUSIONES

CONCLUSIONES

1. La determinación de la actividad catalizada por las enzimas lipoxigenasas codificadas en el genoma de *Arabidopsis*, permite asignar actividad 9-lipoxigenasa a las proteínas codificadas por los genes *LOX1* y *LOX5,* y 13-lipoxigenasa a las codificadas por los genes *LOX2, LOX3, LOX4* y *LOX6*.

2. Los estudios de expresión de los genes *LOXs*, junto con la determinación bioquímica de las oxilipinas que se acumulan en la planta revelan una contribución preferente de las enzimas 9-lipoxigenasa en los tejidos de raíz.

3. La expresión de los genes *9-LOXs*, *LOX1* y *LOX5,* se induce durante la respuesta de *Arabidopsis* a la infección de las bacterias hemibiotrofas *Pseudomonas syringae* pv. *tomato* DC3000 y *Pseudomonas syringae* pv. *tomato* DC3000 *avr*RPM1.

4. La pérdida de la actividad 9-LOXs en el mutante *lox1 lox5* provoca un aumento de la susceptibilidad de la planta frente a la infección de la bacteria virulenta *Pst* DC3000, lo que revela la participación de esta ruta de síntesis de oxilipinas en la activación de la respuesta de defensa de las plantas frente a la infección de este tipo de patógenos.

5. El cartografiado de los mutantes *noxy6* y *noxy22*, insensibles a la acción del 9-HOT, ha permitido localizar dichas mutaciones en los genes *CTR1*

(CONSTITUTIVE ETHYLENE RESPONSE1) y *ETO1* (ETHYLENE-OVERPRODUCER1), respectivamente, que codifican proteínas que actúan como reguladores negativos de la respuesta a etileno (ET).

6. La caracterización de los mutantes *noxy6* y *noxy22*, renombrados *ctr1-15* y *eto1-14*, respectivamente, ha puesto de manifiesto que la producción constitutiva de etileno interfiere en la acción del 9-HOT y que, a su vez, el 9-HOT interfiere con la activación de la señalización regulada por etileno, por lo que ambas señales, 9-HOT y ET ejercen un efecto antagónico y recíproco.

7. La producción constitutiva de etileno interfiere con la activación de la defensa vegetal. Además, al igual que en el mutante *lox1 lox5*, la susceptibilidad del mutante *eto1-14* está asociada a una alteración en la acumulación de especies reactivas de oxígeno en los tejidos infectados.

8. El análisis de la respuesta de las plantas *lox1 lox5* y *eto1-14* (insensibles a 9-HOT) a distintos tipos de estrés oxidativo, revela la participación de las enzimas 9-LOX y del ET en el control de la respuesta de la planta a la generación de oxígeno singlete, en donde la falta de actividad 9-LOX o el defecto en su señalización, aumenta la susceptibilidad frente a este tipo de especies reactivas de oxígeno.

9. El análisis transcripcional de los mutantes *lox1 lox5* y *eto1-14* a la generación de oxígeno singlete, permite confirmar la participación de las 9-LOX y del ET en la regulación de este tipo de estrés oxidativo, en donde ambas rutas controlan una parte importante de dicha respuesta.

10. Los resultados de este trabajo indican que la susceptibilidad de los mutantes *lox1 lox5* y *eto1-14* sería la consecuencia de un defecto en la regulación del estrés oxidativo generado durante la infección de patógenos, cuyo control es esencial para la activación de una respuesta de defensa eficaz, con capacidad para limitar el crecimiento de los patógenos hemibiotrofos en la planta.

BIBLIOGRAFÍA

VARIOS

BIBLIOGRAFÍA

Alonso JM & Stepanova AN (2003) T-DNA mutagenesis in Arabidopsis. *Methods Mol Biol* **236**:177-188.

Alvarez ME (2000) Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant molecular biology* **44**(3):429-442.

Alvarez ME*, et al.* (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92**(6):773-784.

Angelos MG*, et al.* (2006) Hypoxic reperfusion of the ischemic heart and oxygen radical generation. *American journal of physiology. Heart and circulatory physiology* **290**(1):H341-347.

Apel K & Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual review of plant biology* **55**:373-399.

Bannenberg G, Martinez M, Hamberg M, & Castresana C (2009) Diversity of the enzymatic activity in the lipoxygenase gene family of Arabidopsis thaliana. *Lipids* **44**(2):85-95.

Barnett J*, et al.* (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochimica et biophysica acta* **1209**(1):130-139.

Bechtold N & Pelletier G (1998) In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. *Methods Mol Biol* **82**:259-266.

Bevan M (1984) Binary Agrobacterium vectors for plant transformation. *Nucleic acids research* **12**(22):8711-8721.

Broekaert WF, Delaure SL, De Bolle MF, & Cammue BP (2006) The role of ethylene in host-pathogen interactions. *Annual review of phytopathology* **44**:393-416.

Browse J (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual review of plant biology* **60**:183-205.

Carol RJ*, et al.* (2005) A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. *Nature* **438**(7070):1013-1016.

Christians MJ*, et al.* (2009) The BTB ubiquitin ligases ETO1, EOL1 and EOL2 act collectively to regulate ethylene biosynthesis in Arabidopsis by controlling type-2 ACC synthase levels. *The Plant journal : for cell and molecular biology* **57**(2):332-345.

Clarke JD, Aarts N, Feys BJ, Dong X, & Parker JE (2001) Constitutive disease resistance requires EDS1 in the Arabidopsis mutants cpr1 and cpr6 and is partially EDS1-dependent in cpr5. *The Plant journal : for cell and molecular biology* **26**(4):409-420.

Cornejo-Garcia JA*, et al.* (2006) Anti-oxidant enzyme activities and expression and oxidative damage in patients with non-immediate reactions to drugs. *Clinical and experimental immunology* **145**(2):287-295.

Debener T, Lehnackers H, Arnold M, & Dangl JL (1991) Identification and molecular mapping of a single Arabidopsis thaliana locus determining resistance to a phytopathogenic Pseudomonas syringae isolate. *The Plant journal : for cell and molecular biology* **1**(3):289-302.

Deblaere R*, et al.* (1985) Efficient octopine Ti plasmid-derived vectors for Agrobacterium-mediated gene transfer to plants. *Nucleic acids research* **13**(13):4777-4788.

Dellaporta SL*, et al.* (1984) Endogenous transposable elements associated with virus infection in maize. *Cold Spring Harbor symposia on quantitative biology* **49**:321-328.

Dong X (2004) NPR1, all things considered. *Current opinion in plant biology* **7**(5):547-552.

F Gubler AA, A Bacic, AB Blakeney and BA Stone (1985) Release of Ferulic Acid Esters From Barley Aleurone. II. Characterization of the Feruloyl Compounds Released in Response to GA3 *Australian Journal of Plant Physiology* **12**((3)):307 - 317.

Feys BJ, Moisan LJ, Newman MA, & Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *The EMBO journal* **20**(19):5400-5411.

Fonseca S*, et al.* (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nature chemical biology* **5**(5):344-350.

Gechev TS, Van Breusegem F, Stone JM, Denev I, & Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays : news and reviews in molecular, cellular and developmental biology* **28**(11):1091-1101.

Gobel C, Feussner I, Hamberg M, & Rosahl S (2002) Oxylipin profiling in pathogen-infected potato leaves. *Biochimica et biophysica acta* **1584**(1):55-64.

Grechkin AN, Fazliev FN, & Mukhtarova LS (1995) The lipoxygenase pathway in garlic (Allium sativum L.) bulbs: detection of the novel divinyl ether oxylipins. *FEBS letters* **371**(2):159-162.

Guo H & Ecker JR (2003) Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* **115**(6):667-677.

Guzman P & Ecker JR (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *The Plant cell* **2**(6):513-523.

Hamberg M, Ponce de Leon I, Rodriguez MJ, & Castresana C (2005) Alpha-dioxygenases. *Biochemical and biophysical research communications* **338**(1):169-174.

Hanahan D & Meselson M (1983) Plasmid screening at high colony density. *Methods in enzymology* **100**:333-342.

Hematy K, Cherk C, & Somerville S (2009) Host-pathogen warfare at the plant cell wall. *Current opinion in plant biology* **12**(4):406-413.

Hwang IS & Hwang BK (2010) The pepper 9-lipoxygenase gene CaLOX1 functions in defense and cell death responses to microbial pathogens. *Plant physiology* **152**(2):948-967.

Innes RW*, et al.* (1993) Identification of a disease resistance locus in Arabidopsis that is functionally homologous to the RPG1 locus of soybean. *The Plant journal : for cell and molecular biology* **4**(5):813-820.

J. Sambrook EFF, T. Maniatis (1989) Molecular cloning : a laboratory manual.

Jefferson RA, Kavanagh TA, & Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO journal* **6**(13):3901-3907.

Jones JD & Dangl JL (2006) The plant immune system. *Nature* **444**(7117):323-329.

Kachroo A & Kachroo P (2009) Fatty Acid-derived signals in plant defense. *Annual review of phytopathology* **47**:153-176.

Kendrick MD & Chang C (2008) Ethylene signaling: new levels of complexity and regulation. *Current opinion in plant biology* **11**(5):479-485.

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, & Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis,

encodes a member of the raf family of protein kinases. *Cell* **72**(3):427-441.

Koo AJ & Howe GA (2009) The wound hormone jasmonate. *Phytochemistry* **70**(13-14):1571-1580.

Laloi C, Apel K, & Danon A (2004) Reactive oxygen signalling: the latest news. *Current opinion in plant biology* **7**(3):323-328.

Laloi C*, et al.* (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **104**(2):672-677.

Lamb C & Dixon RA (1997) The Oxidative Burst in Plant Disease Resistance. *Annual review of plant physiology and plant molecular biology* **48**:251-275.

Lehti-Shiu MD, Zou C, Hanada K, & Shiu SH (2009) Evolutionary history and stress regulation of plant receptor-like kinase/pelle genes. *Plant physiology* **150**(1):12-26.

Liechti R & Farmer EE (2006) Jasmonate biochemical pathway. *Science's STKE : signal transduction knowledge environment* **2006**(322):cm3.

Loake G & Grant M (2007) Salicylic acid in plant defence--the players and protagonists. *Current opinion in plant biology* **10**(5):466-472.

Loeffler C*, et al.* (2005) B1-phytoprostanes trigger plant defense and detoxification responses. *Plant physiology* **137**(1):328-340.

Logemann J, Schell J, & Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. *Analytical biochemistry* **163**(1):16-20.

Lopez MA, Bannenberg G, & Castresana C (2008) Controlling hormone signaling is a plant and pathogen challenge for growth and survival. *Current opinion in plant biology* **11**(4):420-427.

Lopez MA*, et al.* (2011) Antagonistic role of 9-lipoxygenase-derived oxylipins and ethylene in the control of oxidative stress, lipid peroxidation and plant defence. *The Plant journal : for cell and molecular biology* **67**(3):447-458.

Lorenzo O, Piqueras R, Sanchez-Serrano JJ, & Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *The Plant cell* **15**(1):165-178.

Luckow VA, Lee SC, Barry GF, & Olins PO (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. *Journal of virology* **67**(8):4566-4579.

Malamy JE & Benfey PN (1997) Organization and cell differentiation in lateral roots of Arabidopsis thaliana. *Development* **124**(1):33-44.

Melotto M, Underwood W, Koczan J, Nomura K, & He SY (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**(5):969-980.

Mene-Saffrane L*, et al.* (2009) Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in Arabidopsis. *The Journal of biological chemistry* **284**(3):1702-1708.

Montillet JL*, et al.* (2005) Fatty acid hydroperoxides and H2O2 in the execution of hypersensitive cell death in tobacco leaves. *Plant physiology* **138**(3):1516-1526.

Mueller S*, et al.* (2008) General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in Arabidopsis. *The Plant cell* **20**(3):768-785.

Nanda AK, Andrio E, Marino D, Pauly N, & Dunand C (2010) Reactive oxygen species during plant-microorganism early interactions. *Journal of integrative plant biology* **52**(2):195-204.

Navarro L*, et al.* (2008) DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Current biology : CB* **18**(9):650-655.

Olmedo G*, et al.* (2006) ETHYLENE-INSENSITIVE5 encodes a 5'-->3' exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2. *Proceedings of the National Academy of Sciences of the United States of America* **103**(36):13286-13293.

Onkokesung N*, et al.* (2010) Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in Nicotiana attenuata leaves. *Plant physiology* **153**(2):785-798.

Peltier JB, Ytterberg AJ, Sun Q, van Wijk KJ (2004) New functions of the thylakoid membrane proteome of Arabidopsis thaliana revealed by a simple, fast and versatile fractionation strategy. J Biol Chem 279:49367–49383.

Penninckx IA, Thomma BP, Buchala A, Metraux JP, & Broekaert WF (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *The Plant cell* **10**(12):2103-2113.

Polkowska-Kowalczyk L*, et al.* (2008) Changes in the initial phase of lipid peroxidation induced by elicitor from Phytophthora infestans in Solanum species. *Journal of plant physiology* **165**(18):1929-1939.

Przybyla D*, et al.* (2008) Enzymatic, but not non-enzymatic, 1O2-mediated peroxidation of polyunsaturated fatty acids forms part of the EXECUTER1-dependent stress response program in the flu mutant of Arabidopsis thaliana. *The Plant journal : for cell and molecular biology* **54**(2):236-248.

Qi Y, Tsuda K, Glazebrook J, & Katagiri F (2011) Physical association of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) immune receptors in Arabidopsis. *Molecular plant pathology* **12**(7):702-708.

Robert-Seilaniantz A, Navarro L, Bari R, & Jones JD (2007) Pathological hormone imbalances. *Current opinion in plant biology* **10**(4):372-379.

Rusterucci C*, et al.* (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. *The Journal of biological chemistry* **274**(51):36446-36455.

Saiki RK*, et al.* (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**(4732):1350-1354.

Sanz A, Moreno JI, & Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. *The Plant cell* **10**(9):1523-1537.

Schwessinger B & Zipfel C (2008) News from the frontline: recent insights into PAMP-triggered immunity in plants. *Current opinion in plant biology* **11**(4):389-395.

Spoel SH*, et al.* (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* **137**(5):860-872.

Staswick PE (2008) JAZing up jasmonate signaling. *Trends in plant science* **13**(2):66-71.

Tada Y*, et al.* (2008) Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. *Science* **321**(5891):952-956.

Thomma BP, Nurnberger T, & Joosten MH (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *The Plant cell* **23**(1):4-15.

Thordal-Christensen H (2003) Fresh insights into processes of nonhost resistance. *Current opinion in plant biology* **6**(4):351-357.

Torres MA, Jones JD, & Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. *Plant physiology* **141**(2):373-378.

Triantaphylides C & Havaux M (2009) Singlet oxygen in plants: production, detoxification and signaling. *Trends in plant science* **14**(4):219-228.

Tsuda K & Katagiri F (2010) Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Current opinion in plant biology* **13**(4):459-465.

Van Breusegem F, Bailey-Serres J, & Mittler R (2008) Unraveling the tapestry of networks involving reactive oxygen species in plants. *Plant physiology* **147**(3):978-984.

Vellosillo T*, et al.* (2007) Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. *The Plant cell* **19**(3):831-846.

Vicente J*, et al.* (2011) Role of 9-Lipoxygenase and alpha-Dioxygenase Oxylipin Pathways as Modulators of Local and Systemic Defense. *Molecular plant*.

Vick BA & Zimmerman DC (1983) The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. *Biochemical and biophysical research communications* **111**(2):470-477.

Vlot AC, Dempsey DA, & Klessig DF (2009) Salicylic Acid, a multifaceted hormone to combat disease. *Annual review of phytopathology* **47**:177-206.

Wang KL, Yoshida H, Lurin C, & Ecker JR (2004) Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. *Nature* **428**(6986):945-950.

Whalen MC, Innes RW, Bent AF, & Staskawicz BJ (1991) Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *The Plant cell* **3**(1):49-59.

Woeste KE, Ye C, & Kieber JJ (1999) Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. *Plant physiology* **119**(2):521-530.

Wu J & Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. *Annual review of genetics* **44**:1-24.

Yanofsky MF & Nester EW (1986) Molecular characterization of a host-range-determining locus from Agrobacterium tumefaciens. *Journal of bacteriology* **168**(1):244-250.

Yoo SD, Cho Y, & Sheen J (2009) Emerging connections in the ethylene signaling network. *Trends in plant science* **14**(5):270-279.

Yoshioka H, Asai S, Yoshioka M, & Kobayashi M (2009) Molecular mechanisms of generation for nitric oxide and reactive oxygen species,

and role of the radical burst in plant immunity. *Molecules and cells* **28**(4):321-329.

Zander M, Chen S, Imkampe J, Thurow C, & Gatz C (2011) Repression of the Arabidopsis thaliana Jasmonic Acid/Ethylene-Induced Defense Pathway by TGA-Interacting Glutaredoxins Depends on Their C-Terminal ALWL Motif. *Molecular plant*.

Zhang J*, et al.* (2010) Effector-triggered and pathogen-associated molecular pattern-triggered immunity differentially contribute to basal resistance to Pseudomonas syringae. *Molecular plant-microbe interactions : MPMI* **23**(7):940-948.

Zhang J & Zhou JM (2010) Plant immunity triggered by microbial molecular signatures. *Molecular plant* **3**(5):783-793.

Zhu Z*, et al.* (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **108**(30):12539-12544.

Zimmerli L, Metraux JP, & Mauch-Mani B (2001) beta-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus Botrytis cinerea. *Plant physiology* **126**(2):517-523.

VARIOS

The Plant Cell, Vol. 19: 831–846, March 2007, www.plantcell.org © 2007 American Society of Plant Biologists

Oxylipins Produced by the 9-Lipoxygenase Pathway in *Arabidopsis* Regulate Lateral Root Development and Defense Responses through a Specific Signaling Cascade [W]

Tamara Vellosillo,[a] Marta Martínez,[a] Miguel Angel López,[a] Jorge Vicente,[a] Tomas Cascón,[a] Liam Dolan,[b] Mats Hamberg,[c] and Carmen Castresana[a,1]

[a] Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma, Cantoblanco, E-28049 Madrid, Spain

[b] Cell and Developmental Biology Department, John Innes Centre, Colney, NR4 7UH Norwich, United Kingdom

[c] Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden

Arabidopsis thaliana **seedling growth with pure oxylipins resulted in root waving, loss of root apical dominance, and decreased root elongation. 9-Hydroxyoctadecatrienoic acid (9-HOT) was a potent inducer of root waving. Studies with *noxy2* (for *nonresponding to oxylipins2*), a new 9-HOT–insensitive mutant, and *coronatine insensitive1-1* (jasmonate-insensitive) revealed at least three signaling cascades mediating the oxylipin actions. Treatment with 9-HOT resulted in a reduction in lateral roots and an increase in stage V primordia. Roots showed strong 9-lipoxygenase (9-LOX) activity, and root primordia expressed 9-LOX genes. These results, along with findings that *noxy2* and mutants with defective 9-LOX activity showed increased numbers of lateral roots, suggest that 9-HOT, or a closely related 9-LOX product, is an endogenous modulator of lateral root formation. Histochemical and molecular analyses revealed that 9-HOT activated events common to development and defense responses. A subset of 9-HOT–responding root genes was also induced in leaves after 9-HOT treatment or pathogen inoculation. The results that *noxy2* displayed altered root development, enhanced susceptibility to *Pseudomonas*, and reduced the activation of 9-HOT–responding genes are consistent with mechanistic links among these processes. The nature of the changes detected suggests that oxylipins from the 9-LOX pathway function in cell wall modifications required for lateral root development and pathogen arrest.**

INTRODUCTION

The capacity of plants to survive adverse conditions and reach reproductive maturity critically depends on their ability to continuously adapt to changes in the environment. Therefore, plants have evolved an array of intricate regulatory mechanisms that involve the generation of signaling molecules mediating the activation of required adaptive responses. In particular, the activation of pathogen-specific defense mechanisms upon microbial infection, as well as the acquisition of structural and physiological adjustments to environmental changes, permit survival, plant development, and reproduction.

A common group of metabolites playing a fundamental role in the physiological and pathological responses of plants and vertebrates are lipid derivatives generated by oxygenation and further transformation of fatty acids. In mammals, eicosanoids, including prostaglandins and leukotrienes, are generated mainly by the oxygenation of arachidonic acid, and their receptor-mediated roles in various processes, such as the immune response, fever, pain, and inflammation, are well understood. By contrast, information regarding the roles of octadecanoids derived from linoleic and linolenic acids in plants is more limited.

The biosynthesis of plant oxylipins is initiated by the action of lipoxygenases (9-LOX and 13-LOX) or α-dioxygenase (α-DOX), which catalyze the oxygenation of predominantly linoleic acid (18:2) and linolenic acid (18:3) into reactive hydroperoxides. Secondary transformations of these hydroperoxides are catalyzed by allene oxide synthases, divinyl ether synthases, hydroperoxide lyases, peroxygenases, and epoxy alcohol synthase. The oxygenated derivatives thus formed include the phytohormone jasmonic acid (JA) as well as reactive oxylipins possessing epoxide, conjugated carbonyl, or aldehyde functionalities (reviewed in Blée, 2002; Howe and Schilmiller, 2002; Liavonchanka and Feussner, 2006). A schematic representation of the oxylipin metabolic pathways and of the products synthesized is provided in Supplemental Figure 1 online.

LOX-derived oxylipins are involved in physiological processes of plants, such as growth and fertility (Creelman and Mullet, 1997; Sanders et al., 2000; Stintzi and Browse, 2000) and mechanotransduction (Stelmach et al., 1998). In addition, oxylipins have important roles in the adaptation of plants to adverse growth conditions (Staswick et al., 1992; Creelman and Mullet,

[1] To whom correspondence should be addressed. E-mail ccastresana@cnb.uam.es; fax 34-91-5854506.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Carmen Castresana (ccastresana@cnb.uam.es).

[W] Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.106.046052

1995; Armengaud et al., 2004) and in the defense reactions that take place as a consequence of the infection of plants with bacterial and fungal pathogens. Numerous studies aimed at defining the action of oxylipins have shown that the expression of genes encoding the enzymes initiating the synthesis of oxylipins is specifically induced upon inoculation with plant pathogens (Melan et al., 1993; Sanz et al., 1998; Jalloul et al., 2002; Turner et al., 2002). Moreover, alterations in the synthesis of oxylipins in mutants and transgenic lines have been shown to modify the plant response to pathogen infection (Rancé et al., 1998; Vijayan et al., 1998; De León et al., 2002; Farmer et al., 2003).

Analytical approaches have identified the different types of oxylipins produced in plants (Mueller et al., 2006). Furthermore, improvements of synthetic and biosynthetic methodologies provide increasing quantities and numbers of oxylipins for use in biological work (Prost et al., 2005). It is known that oxylipins have antimicrobial effects, stimulate plant defense gene expression, and regulate plant cell death. In vitro studies using pure derivatives showed that oxylipins impair the growth of some plant microbial pathogens, including bacteria, oomycetes, and fungi (Croft et al., 1993; Weber et al., 1999; Prost et al., 2005). On the other hand, many 13-LOX–derived compounds, including the hormone JA, 12-oxophytodienoic acid (OPDA), 13-hydroxyoctadecatrienoic acid (13-HOT), and 13-hydroperoxide lyase–derived C6 aldehydes, are regulators of plant defense gene expression (Bate and Rothstein, 1998; Weichert et al., 1999; Alméras et al., 2003). Also, electrophilic ketodienes (KODs) and ketotrienes (KOTs), derived from linoleic and linolenic acids, respectively, were shown to induce plant defense genes (Vollenweider et al., 2000). Importantly, JA has been shown to be a key defense molecule that interacts with additional defense pathways to control resistance to necrotrophic pathogens (Penninckx et al., 1996; Glazebrook, 2005). Activation of the oxylipin pathways can also lead to the production of molecules regulating plant cell death. Thus, apart from being precursors of numerous other oxylipins, LOX-generated 9- and 13-hydroperoxides have been shown to affect plant cell viability, and a role of hydroperoxides in regulating localized cell death during the hypersensitive reaction has been suggested (Rustérucci et al., 1999). Similarly, fatty acid KODs and KOTs were shown to display cell death–promoting activities (Vollenweider et al., 2000). Also, the α-DOX pathway contributes to plant defense against bacterial infection by controlling the development of the hypersensitive reaction (De León et al., 2002; Hamberg et al., 2003).

Despite increasing experimental evidence, the mechanisms by which oxylipins exert their activity remain essentially unknown. JA is an exception because a number of the components mediating its signaling have been defined recently (for a recent review, see Lorenzo and Solano, 2005). Among those, the F-box protein CORONATINE INSENSITIVE (COI) plays a central role (Xie et al., 1998).

Here, we used a collection of pure oxylipins synthesized through the 13-LOX, 9-LOX, and α-DOX pathways and an in vitro seedling assay to study the functionality of these compounds in physiological and pathological processes. Systematic analyses of plants grown in the presence of oxylipins showed three different phenotypic alterations: root waving with lateral root arrest, growth arrest with loss of root apical dominance, and overall decrease of root elongation. Detailed characterization of the waving response to 9-HOT, a 9-LOX derivative, suggested a role of this oxylipin in both the formation of lateral roots and the defense against pathogens. Moreover, studies of mutants that do not develop the root-waving morphology allowed the definition of the signaling pathways that mediate the cellular responses to oxylipins.

RESULTS

Oxylipins Induce Distinct Developmental Alterations in Roots of *Arabidopsis* Seedlings

Functional analyses using a set of pure and well-characterized oxylipins (see Supplemental Figure 1 online) demonstrated striking effects on root development. As shown in Figure 1, we found that 10 of the oxylipins tested, all possessing hydroxyl and/or keto functionalities (9-HOT, 13-HOT, 13- and 9-hydroxyoctadecadienoic [13- and 9-HOD], 2-hydroxyoctadecenoic acid [2-HOE], 9-ketooctadecatrienoic acid [9-KOT], 13- and 9-ketooctadienoic acid [9- and 13-KOD], 9-hydroxy-10-ketooctadecenoic acid [9,10-KHOE], and 13-hydroxy-12-ketooctadecadienoic [12,13-KHOD]) induced waving of *Arabidopsis thaliana* roots with growth arrest of lateral roots. In addition, another group of six of oxylipins (9-oxononanoic acid [9-oxo-C_9], 12-oxo-12:1(E) [traumatin], colnelenic acid, colneleic acid, JA, and OPDA) was found to arrest root growth. Among these, two phenotypes were observed: loss of apical dominance, with the development of increased numbers of lateral and adventitious roots (induced by 9-oxo-C_9, traumatin, colnelenic acid, and colneleic acid [i.e., short-chain oxoacids or potential precursors of such compounds]); and a general decrease in root elongation, resulting in the formation of shorter roots than in control plants (induced by the cyclic oxylipins JA and OPDA). By contrast, growing plants in the presence of the remaining 28 oxylipins, including hydroperoxides, aldehydes, epoxides, epoxy alcohols, diols, and triols (see Supplemental Figure 1 online) produced no visible root phenotype. Concentrations of oxylipins tested in these analyses are described in Methods, and optimal amounts to induce the phenotypes described are shown in Figure 1.

The group of oxylipins producing root waving are biosynthesized from fatty acid hydroperoxides by allene oxide synthase (9,10-KHOE and 12,13-KHOD), reductase/peroxidase (hydroxy acids), or LOX (keto acids). Concentrations needed for activity ranged from 25 to 75 μM, although 9-HOT was active even at 15 μM (see Supplemental Figure 2 online). Additional results with seedlings grown for 6 d on Murashige and Skoog (MS) medium and then transferred to 9-HOT–containing plates revealed that the waving phenotype was locally restricted to the root zone grown in the presence of inducer (Figure 1).

By contrast, oxylipins producing a root growth–inhibiting effect with loss of apical dominance and increase of lateral roots are biosynthesized from hydroperoxides by either hydroperoxide lyase or divinyl ether synthase. The strongest activity was observed with 9-oxo-C_9 (active at 15 μM), an oxylipin produced from linole(n)ic acid 9-hydroperoxides by hydroperoxide lyase. The third phenotype, overall decreased root elongation, was induced by OPDA and JA at concentrations of 2 and 10 μM,

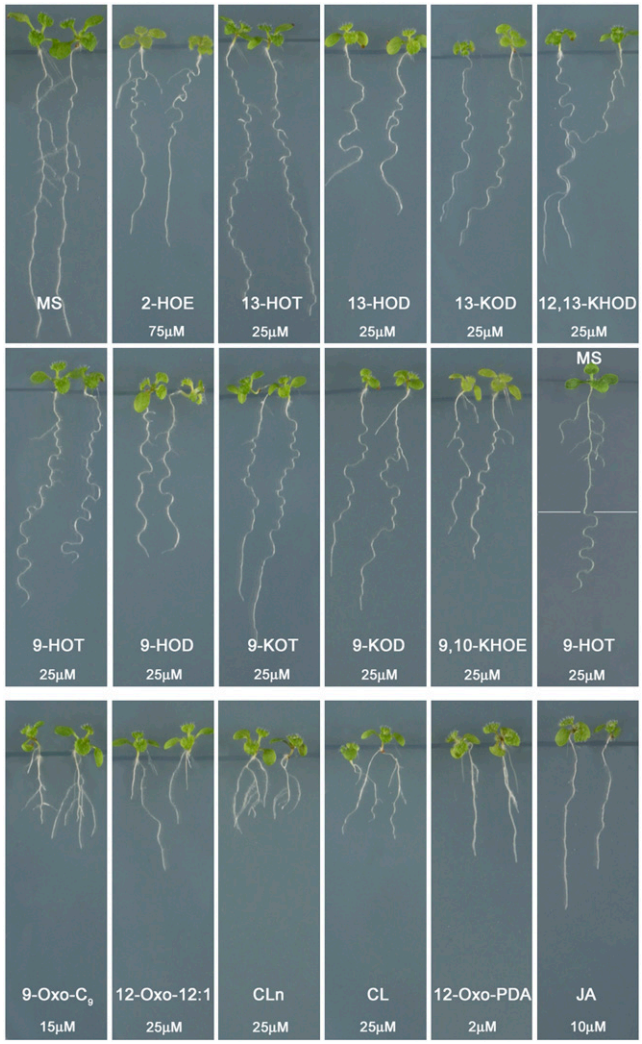


Figure 1. Phenotypic Alterations Provoked in Root of *Arabidopsis* Seedlings by Distinct Oxylipins.

Seedlings were grown vertically on the surface of hard agar plates. Shown are 10-d-old seedlings grown in control MS medium and in medium containing different concentrations of oxylipins. Three types of phenotypes were noted: root waving with lateral root arrest, caused by 2-HOE, 13-HOT, 13-HOD, 13-KOD, 12,13-KHOD, 9-HOT, 9-HOD, 9-KOT, 9-KOD, and 9,10-KHOE; growth arrest with loss of apical dominance, caused by 9-oxo-C_9, 12-oxo-12:1, colnelenic acid (Cln), and colneleic acid (CL); and overall root shortening, caused by OPDA and JA. The last panel in the middle row shows an *Arabidopsis* seedling grown for 6 d in MS medium and then transferred for 4 additional days to a 9-HOT–containing MS medium. Optimal concentrations inducing the phenotypes described are indicated. Complete names of oxylipins are shown in Supplemental Figure 1 online.

respectively. Biosynthesis of these oxylipins takes place from linolenic acid 13-hydroperoxide and involves allene oxide synthase and allene oxide cyclase. Therefore, the three distinct morphological changes are induced by distinct sets of derivatives that are formed from different parts of the oxylipin biosynthetic pathway.

9-HOT Causes Waving of Roots and Inhibits the Emergence of Lateral Roots

The root-waving phenotype was accompanied by an obvious reduction in the number of lateral roots. Lateral root development proceeds through a number of discrete stages designated I to VIII (Malamy and Benfey, 1997). As seen in Figure 2, the profile of primordia of seedlings grown in the presence of 25 μM 9-HOT was shifted toward an increased abundance of early stages (I to V) and a reduced abundance of later stages (VI to VIII and lateral roots). The strongest effect was noted in the number of stage V primordia, which was 2.5- to 3-fold higher in 9-HOT–treated roots compared with control roots. A similar distribution of primordia was observed in roots grown in the presence of 15 or 35 μM 9-HOT, and the abundance of primordia at stage V was consistently higher than that in control roots (percentages of stage V primordia of total primordia: 14, 27, and 10% for roots grown in the presence of 15 and 35 μM 9-HOT or in the absence of 9-HOT, respectively) (see Supplemental Figure 2 online). This finding suggests that 9-HOT treatment results in a blockage of the lateral root development pathway at stage V, whereas early stages of lateral root development are little affected. The dose-dependent response observed is consistent with oxylipins playing a physiological role in the development but not in the initiation of the lateral root primordia (LRP).

LOX1, *LOX3*, and *LOX5* Genes Are Expressed in LRP

To evaluate the functional role of oxylipins during the development of lateral roots, the expression of *LOX* and α-*DOX* genes was examined by means of promoter β-glucuronidase (GUS) constructs (see Methods for a detailed description). Analysis of gene transcription revealed expression in leaves of transgenic seedlings

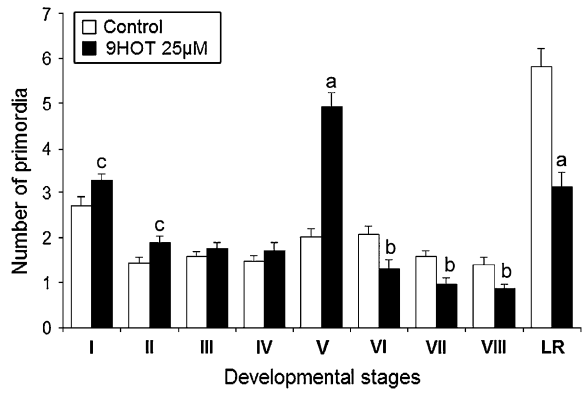


Figure 2. Analyses of LRP in Columbia Wild-Type Plants.

Number of primordia found in roots of 10-d-old *Arabidopsis* seedlings grown on MS medium and on 9-HOT–containing medium. Eight developmental stages and emergent lateral roots were counted. A representative example of the developmental stages examined is shown in Supplemental Figure 3 online. Means and SE are shown. Letters above the bars indicate statistically significant differences between seedlings grown on MS medium and 9-HOT–containing medium (*t* test; $P < 0.001$ [a], $0.001 < P < 0.01$ [b], $0.001 < P < 0.05$ [c]). The data presented are results obtained in five independent experiments.

with α-*DOX2*, *LOX1*, *LOX2*, *LOX3*, and *LOX4* reporter genes and in roots of plants containing α-*DOX1*, *LOX1*, *LOX3*, and *LOX5* constructs, whereas no GUS activity was observed in seedlings containing the *LOX6* reporter gene (Figures 3A to 3H). GUS staining in roots of the α-*DOX1* lines was localized in the epidermal cells, whereas plants expressing the *LOX1*, *LOX3*, and *LOX5* promoters showed staining in LRP (Figure 3). In plants harboring the *LOX1:GUS* construct, GUS activity was detected in the pericycle cells and in the LRP from stages I to VII, from which expression declined at the time of lateral root emergence (Figures 3I to 3L). Expression directed by *LOX3* and *LOX5* was primarily restricted to the LRP, in which case blue staining was detected from the first pericycle divisions. Notably, whereas expression in *LOX5:GUS* plants diminished before root emergence, GUS activity was maintained in lateral roots in *LOX3* plants (Figures 3M to 3T).

The pattern of gene expression characterized is consistent with a role of LOX1, LOX3, and LOX5 in the developmental process leading to the formation of lateral roots. Based on their putative amino acid sequences, LOX1 and LOX5 have been proposed to be 9-LOXs, whereas *LOX3* is likely to encode a 13-LOX isoform (Liavonchanka and Feussner, 2006). These results suggest a role of both 9-LOX– and 13-LOX–generated oxylipins in the development of LRP and also suggest that the 9-LOX products could be primarily associated with the development process preceding root emergence.

Oxygenation of Linolenic Acid in Roots of *Arabidopsis*

Because the exogenous application of 9-HOT alters root development and the *LOX1* and *LOX5* genes are expressed in roots, the 9-LOX activity of roots was examined by incubating linolenic acid with root homogenates from wild-type seedlings. Results from such analyses revealed that linolenic acid (300 μM) was completely metabolized into polar products when incubated with a 1:10 (w/v) homogenate of wild-type *Arabidopsis* roots. LOX products detected by gas chromatography–mass spectrometry (GC-MS) included 9-HOT and 9-KOT as well as epoxy alcohols and trihydroxy acids (see Supplemental Figure 3 online). The

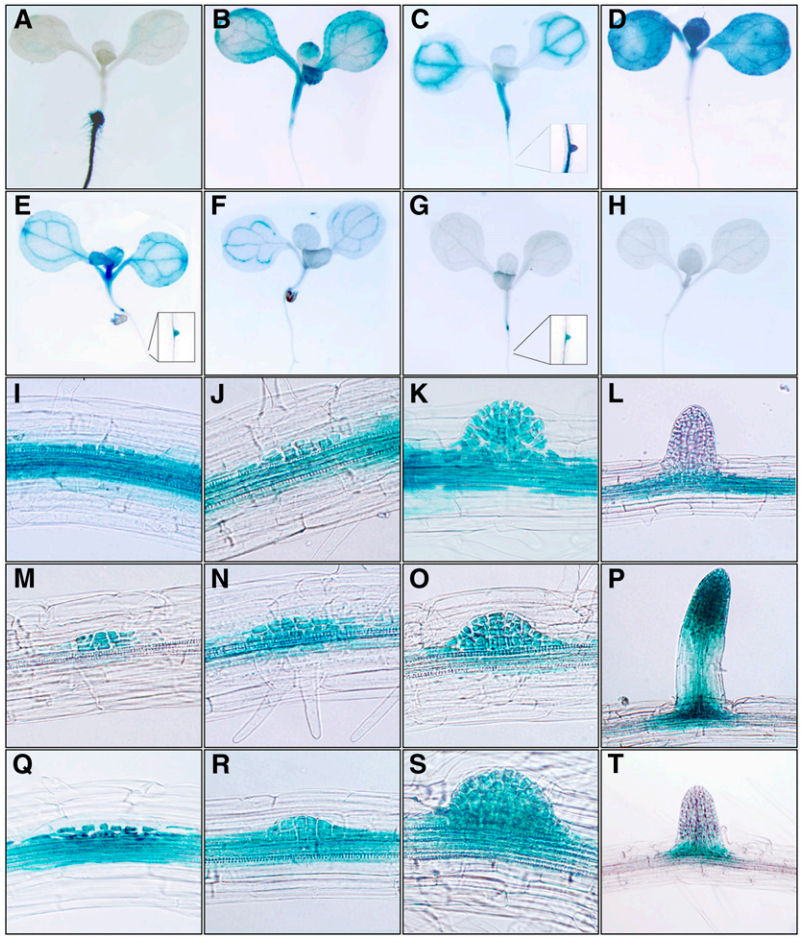


Figure 3. Expression Analyses of α-*DOX* and *LOX* Genes Encoding the Enzymes Initiating the Synthesis of Oxylipins.

Histological examination of GUS activity in seedlings of transgenic lines containing promoter GUS constructs is shown.
(A) to **(H)** GUS staining of seedlings from α-*DOX1:GUS* **(A)**, α-*DOX2:GUS* **(B)**, *LOX1:GUS* **(C)**, *LOX2:GUS* **(D)**, *LOX3:GUS* **(E)**, *LOX4:GUS* **(F)**, *LOX5:GUS* **(G)**, and *LOX6:GUS* **(H)**. Details of the GUS activity found in root primordia are shown for *LOX1:GUS* **(C)**, *LOX3:GUS* **(E)**, and *LOX5:GUS* **(G)**.
(I) to **(T)** Selected phases during the formation of LRP in plants with reporters: *LOX1*, **(I)** to **(L)**; *LOX3*, **(M)** to **(P)**; and *LOX5*, **(Q)** to **(T)**.

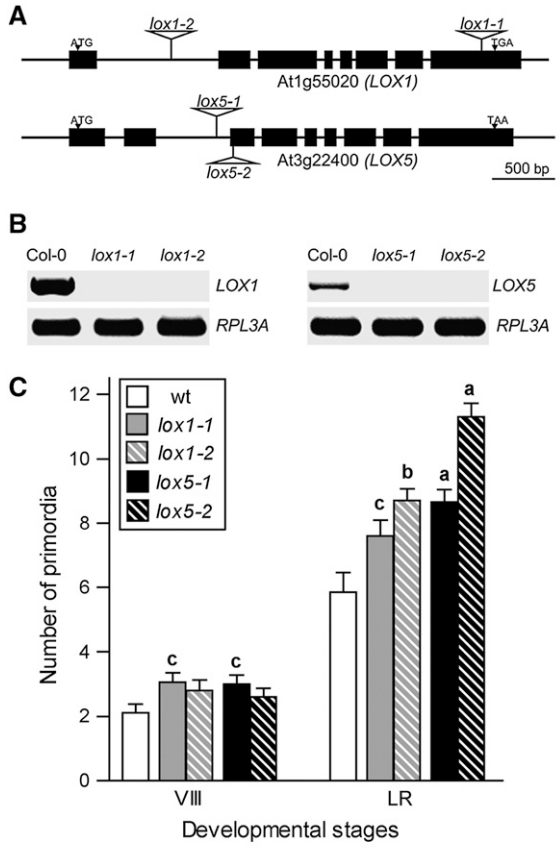
latter two derivatives can be formed as nonspecific degradation products of hydroperoxides accumulating because of low activity of specific hydroperoxide-metabolizing enzymes. That an accumulation of 9-HPOT indeed took place was proven by a separate incubation in which addition of the hydroperoxide reductant $SnCl_2$ inhibited the appearance of degradation products and at the same time strongly enhanced the recovery of the hydroperoxide reduction product 9-HOT. Additional compounds detected in these studies were the α-DOX products 8,11,14-heptadecatrienal and 2-HOT as well as an α-DOX–9-LOX double oxygenation product tentatively identified as 2-hydroxy-9-KOT. Interestingly, 13-LOX products were not observed in these incubations. The 9-LOX activity found in root homogenates is consistent with a role of this oxylipin pathway in root development, in which the 9-LOX derivatives produced might regulate the emergence of lateral roots.

Mutation of *LOX1* and *LOX5* Increases the Number of Lateral Roots

Given that *LOX1* and *LOX5* are expressed in LRP and that treatment of wild-type plants with 9-HOT represses lateral root development, we predicted that plants lacking LOX1 and LOX5 function should develop more lateral roots than wild-type plants. Therefore, we examined the phenotypes of plants lacking 9-LOX function for defective lateral root phenotypes. Homozygous T-DNA insertion mutants that lacked LOX1 (*lox1-1*) and LOX5 (*lox5-1*) function developed more emergent (stage VIII) and lateral roots than wild-type plants at 10 d, as predicted (Figure 4), whereas no significant differences were observed at the remaining developmental stages (I to VII) (see Supplemental Figure 4 online). Analyses of two additional T-DNA insertion lines, *lox1-2* and *lox5-2*, further supported our results showing that the lack of LOX1 and LOX5 function led to an increment in the number of lateral roots (Figure 4). In addition, a moderate increase in the length of the primary root was observed in the *lox1* and *lox5* mutants examined, which thus maintained the overall density of primordia as in roots of wild-type plants (Figure 4). This finding suggests that the effect of the *lox1* and *lox5* mutations is not to increase lateral root initiation but to promote the number of lateral roots. Therefore, these analyses indicated that LOX1 and LOX5 may function as regulators of root development by controlling the emergence of lateral roots through the production of 9-HOT.

9-HOT Induces the Formation of Polysaccharide Deposits and the Production of Reactive Oxygen Species in Waving Roots and in Leaves

Further examination using specific dyes revealed that the root-waving response induced by 9-HOT was accompanied by the accumulation of callose, a high-molecular-weight β-1,3-glucan that plays a role in development and in the response of plants to biotic and abiotic stress (Verma and Hong, 2001). As shown in Figure 5, aniline blue staining revealed deposits of callose that were frequently located along the concave face of the waves. Visualization of root transverse sections revealed that callose deposits were present in two or three closely located epidermal cells per section (Figure 5B), clearly visible from the root surface



D

Genotypes	Total Primordia	Primary Root Length (cm)	Primordia Density
wt	20.4 ± 0.7	3.1 ± 0.08	6.58 ± 0.24
lox1-1	22.6 ± 1.1	3.2 ± 0.09	7.16 ± 0.49
lox1-2	22.3 ± 0.9	3.4 ± 0.07	6.59 ± 0.27
lox5-1	24.7 ± 0.8	3.8 ± 0.04	6.48 ± 0.19
lox5-2	25.9 ± 0.9	3.9 ± 0.05	6.64 ± 0.19

Figure 4. Analyses of LRP in Columbia Wild-Type Plants and in *lox1* and *lox5* Mutants.

(A) Scheme of *LOX1* and *LOX5* genomic structures with exons represented as black boxes. The positions of the T-DNA insertions are indicated.

(B) RT-PCR of RNA isolated from leaves of the genotypes indicated. Gene At1g43170 encoding the 60S ribosomal protein L3 (RPL3A) was used to normalize transcript levels in each sample. Gene-specific primer sets used for the evaluation of RNA are shown in Supplemental Table 2 online.

(C) Number of emergent (VIII) and lateral roots (LR) found in roots of 10-d-old wild-type *Arabidopsis* seedlings and *lox1-1*, *lox1-2*, *lox5-1*, and *lox5-2* mutants grown on MS medium. Means and SE are shown. The data presented are results obtained in three independent experiments. Letters above the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (*t* test; $P < 0.001$ [a], $0.001 < P < 0.01$ [b], $0.001 < P < 0.05$ [c]).

(D) Number of total root primordia, length of the primary root, and lateral root density in 10-d-old wild-type *Arabidopsis*, *lox1-1*, *lox1-2*, *lox5-1*, and *lox5-2* seedlings grown on MS medium. Means and SE of measurements of 20 seedlings are shown.

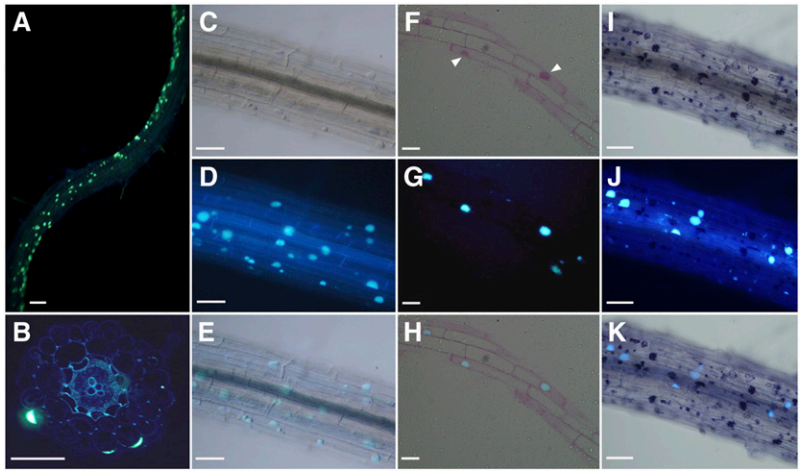


Figure 5. Histological Examination of 9-HOT–Induced Waved Roots.

(A), **(D)**, **(G)**, and **(J)** Fluorescence visualization of callose deposits observed in 9 HOT roots stained with aniline blue. All bars = 50 μm.
(B) Transverse section of an aniline blue–stained root.
(C) Nomarski image of a 9-HOT–treated root.
(E) Merging of images **(C)** and **(D)**.
(F) Transmitted light microscopic visualization of root sections stained with ruthenium red. Pectin deposits are indicated by arrowheads.
(H) Merging of images **(F)** and **(G)**.
(I) Transmitted light microscopic visualization of ROS in roots stained with NBT.
(K) Merging of images **(I)** and **(J)**.

as cellular vesicles (Figures 5C to 5E). In addition to aniline blue staining, accumulation of callose in the 9-HOT–treated roots was determined using a specific anti-callose antibody. Both markers (aniline blue and anti-callose antibodies) stained the same cellular structures, unambiguously establishing the identity as callose (see Supplemental Figure 5 online). In contrast with 9-HOT–treated roots, no callose deposits were detected in roots of control untreated plants (see Supplemental Figure 5 online).

In addition to β-1,3-glucan, the use of ruthenium red revealed the presence of pectin colocalizing with the callose, showing that these deposits were infiltrated by additional polysaccharides (Figures 5F to 5H). Furthermore, staining of waved roots with nitroblue tetrazolium (NBT) revealed a strong production of reactive oxygen species (ROS), visualized as blue formazan deposits formed by the reduction of NBT in the presence of superoxide ions (Figure 5I). The accumulation of ROS was not restricted to the callose-containing vesicles; instead, abundant NBT precipitates were found dispersed along the roots (Figures 5J and 5K), with staining as its most intense in the meristematic and elongation zones.

Challenging leaves with 9-HOT resulted in callose accumulation and ROS production, as it did in roots. Detection of ROS was maximal at 30 min after treatment, whereas the formation of callose deposits started at ~8 h after treatment and increased to its highest accumulation at 24 h (Figure 6). Moreover, ROS were observed homogeneously distributed through the leaf area infiltrated, whereas callose was found dispersed, forming small deposits. A weak accumulation of callose and production of ROS were found in leaves treated with the 9-HOT precursor linolenic acid, whereas both responses were nearly undetectable in untreated leaves or in leaves infiltrated with water, used as controls in these experiments.

Identification of Genes Responding to 9-HOT Treatment

To gain a further understanding of the cellular responses activated in roots when growing in the presence of 9-HOT, changes

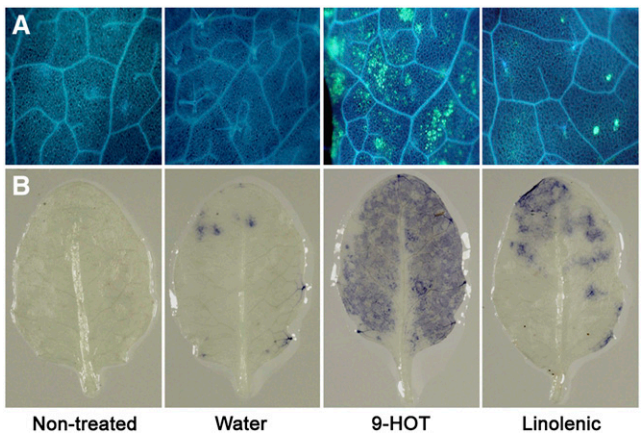
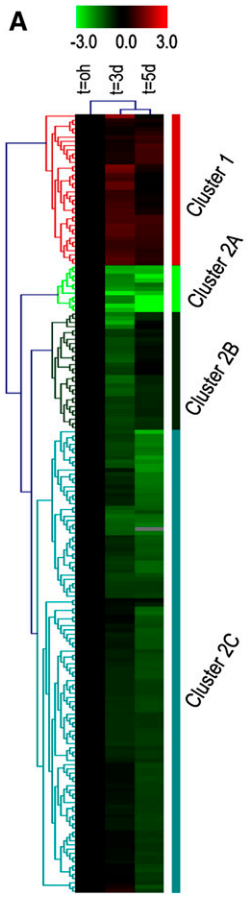


Figure 6. Analyses of Callose Accumulation and ROS Production in Leaves of 4-Week-Old *Arabidopsis* Plants.

Representative examples of treated leaves are shown.
(A) Aniline blue staining of nontreated leaves and leaves infiltrated with water, 9-HOT, or linolenic acid.
(B) NBT staining in leaf samples treated as described for **(A)**.

in gene expression were examined by microarray analysis. Transcriptional profiles of RNA samples from roots after treatment during 3 and 5 d with 25 μM 9-HOT were compared with those of roots growing in the absence of 9-HOT for the same periods (see plant treatments in Methods for a detailed description). For each time point, three separate microarrays were hybridized using RNA extracted from three independent biological replicates. Complete microarray data sets obtained were analyzed, and a list of differentially expressed genes is shown in Supplemental Table 1 online. Details of computational methods to process gene expression data are described in Methods. Of the 26,173 genes represented on the microarray (Galbraith et al., 2004), 178 showed altered expression, with genes induced by 9-HOT (cluster 1, containing 34 genes) and genes repressed by 9-HOT (cluster 2, containing 144 genes). Moreover, three subclusters, 2A, 2B, and 2C, were defined according to the grade and the timing of suppression (Figure 7A). Genes from clusters 1 and 2A, upregulated and downregulated, respectively, were selected for further examination by semiquantitative RT-PCR using specific primers and RNA samples from control and 9-HOT–treated roots (Figure 7B; see Supplemental Table 2 online). Furthermore, because the 9-LOX oxylipin pathway is activated in leaves responding to bacterial infection (Hamberg et al., 2003), gene expression was examined in leaves subjected to 9-HOT treatment as well as in leaves of plants responding to the avirulent bacterium *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 *avrRpm1* and to the virulent strain *Pst* DC3000.

As shown in Figure 7C, significant levels of transcripts were detected for most genes (10 of 11) in control roots that varied according to the microarray data in the 9-HOT–treated samples. The nature of the genes suggested that 9-HOT treatment altered the expression of genes encoding proteins involved in oxidative stress and modifications of the cell wall (Figure 7B). Basal gene expression was much lower in leaves. Approximately 30% of the



B

Locus	Description	Ratio 3 days	Ratio 5 days
At5g22140	Pyridine nucleotide-disulphide oxidoreductase family protein	2.160	1.756
At5g38020	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	2.120	1.565
At1g05560	UDP-glucose transferase (UGT75B2)	1.939	1.847
At5g64120	Peroxidase, putative	1.864	1.658
At1g53830	Pectinesterase family protein	1.277	1.858
At5g09530	Hydroxyproline-rich glycoprotein family protein	1.336	1.821
At5g10230	Annexin 7 (ANN7)	1.330	1.856
At2g30750	Cytochrome P450 71A12, putative	-6.204	-8.446
At1g02920	Glutathione S-transferase, putative	-4.213	-4.795
At4g14630	Germin-like protein (GLP9)	-3.896	-3.350
At1g26390	FAD-binding domain-containing protein	-3.303	-8.578

C

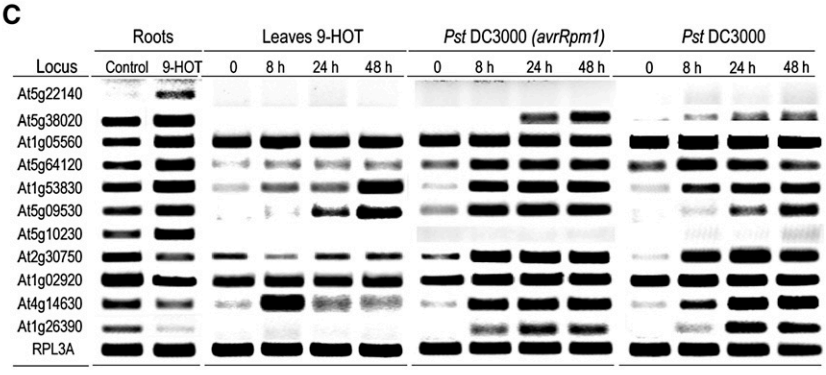


Figure 7. Identification of Genes Responding to 9-HOT and Analyses of Gene Expression.

(A) Cluster analysis of genes changing their expression in roots treated with 9-HOT. Cluster 1 contains upregulated genes. Clusters 2A, 2B, and 2C are highly, slightly, and moderately downregulated genes, respectively. A complete list is provided in Supplemental Table 1 online.

(B) List of genes selected from microarray data for further characterization. Shown are locus designations, descriptions of putative encoded proteins, and differential expression ratios between roots grown in the absence of 9-HOT and roots grown for 3 or 5 d in 9-HOT–containing medium.

(C) RT-PCR analysis of genes in control and 9-HOT–treated roots, leaves subjected to 9-HOT treatment, and leaves responding to inoculation with the avirulent bacterium *Pst* DC3000 *avrRpm1* or the virulent strain *Pst* DC3000. Gene At1g43170 encoding RPL3A was used to normalize transcript levels in each sample.

upregulated genes in roots were also activated in 9-HOT–treated leaves, and this proportion increased to 57% in leaves responding to bacterial inoculation. Remaining root upregulated genes were either not expressed in leaves (At5g22140 and At5g10230) or did not alter its expression during these treatments (see At1g05560 in 9-HOT infiltration and in bacterial inoculation). Notably, a significant proportion of the genes downregulated in roots were induced in 9-HOT leaves (25%) and in bacteria-treated leaves (75%). An explanation for this expression pattern may be that the differences in basal expression observed in roots versus leaves are likely to reflect a variation in the production of endogenous signal(s) mediating expression in these two tissue types. Also, additional regulatory pathways might interact differentially in roots and leaves with the 9-HOT response. Further analyses of gene expression are being performed to investigate these possibilities.

Isolation of Mutants Not Responding to 9-HOT

The root-waving phenotype characterized here was used to screen for mutants that are insensitive to 9-HOT. From a screen of ~20,000 M2 seeds, 18 putative mutants, designated *noxy* (for *nonresponding to oxylipins*), failed to induce root waving, and one of them, *noxy2*, was selected for further characterization in this article (Figure 8). The genetic nature of the *noxy2* mutation was examined in F1 and F2 populations generated from a backcross with wild-type plants. Results from these analyses indicated that the *noxy2* mutation segregated as a single recessive trait. For mapping, *noxy2* was outcrossed to the C-24 ecotype. Molecular markers polymorphic between these two ecotypes defined the position of *noxy2* on chromosome 5, flanked by simple sequence length polymorphisms nga249 and ca72. Generation of additional markers based on the information from the Marker Tracker and MASC single nucleotide polymorphism databases delimited *NOXY2* to a 400-kb region between markers N5-3597960 and MASC04596. As shown in Figure 8A, the *noxy2* mutant failed to form root waves when grown on 9-HOT–containing plates. Analyses of callose accumulation in response to 9-HOT showed that plugs were almost absent. Furthermore, ROS production in roots, as determined by NBT staining, was diminished compared with that in wild-type plants (Figure 8A). Finally, the *noxy2* mutant showed a significant increase in the formation of lateral roots (Figure 8B).

NOXY2 Contributes to Plant Defense

Because roots of *noxy2* were insensitive to 9-HOT and the 9-LOX oxylipin pathway is activated in leaves responding to bacterial infection (Hamberg et al., 2003), plants lacking NOXY2 function might respond differently than wild-type plants to pathogen infection. Therefore, we examined the response of *noxy2* to the bacterial strains *Pst* DC3000 *avrRPM1* (avirulent) and *Pst* DC3000 (virulent). As seen in Figure 8, control plants treated with *Pst* DC3000 *avrRPM1* showed a typical hypersensitive reaction necrotic response in the area inoculated. This response varied significantly in *noxy2* mutants. In this line, leaves developed chlorotic symptoms of variable size that were most prominent between leaf veins. Symptoms described appeared simultaneously in both types of plants and were clearly distinguished at 48 h after inoculation with a bacterial suspension of 10^6 colony-forming units (cfu)/mL (Figure 8C). Significant differences in symptom development were also apparent after using a low-dose inoculum (10^5 cfu/mL) of *Pst* DC3000. A light yellow area was observed in wild-type plants at 48 h after bacterial inoculation, whereas a more intense chlorotic zone was formed in *noxy2* mutants (Figure 8C). As infection progressed, control plants developed a necrotic zone surrounded by a chlorotic halo, characteristic of *Pst* DC3000, whereas chlorotic symptoms were primarily observed in *noxy2* plants. The variation in the formation of symptoms diminished when plants were treated with a high-dose inoculum (10^7 cfu/mL).

Further investigation of NOXY2 function was performed by measuring in planta bacterial growth and expression of both well-known defense markers and the 9-HOT–responsive genes identified in this study (Figure 7). As shown in Figure 8D, higher growth rates were found in *noxy2* mutants. The growth of *Pst* DC3000 *avrRPM1* was increased ~5-fold in *noxy2* relative to control plants, whereas the growth of *Pst* DC3000 increased 10-fold in *noxy2* mutants with respect to wild-type plants. Enhanced bacterial growth in *noxy2* mutants was accompanied by a delayed and reduced accumulation of transcripts from the salicylic acid–responsive genes *PR1* and *PR2*. Also, the accumulation of transcripts from the 9-HOT–responsive genes (At5g64120, A1g09530, At2g30750, and At4g14630) was decreased in *noxy2* mutants, in which lower levels of RNA corresponded to leaves inoculated with the virulent bacterium. As with these genes, expression of the JA and ethylene marker gene *PR4* was preferentially reduced in *noxy2* plants after *Pst* DC3000 inoculation (Figure 8E). Finally, examination of At1g05560 and At1g02920 (upregulated in 9-HOT–treated roots but whose expression did not vary in leaves of control plants subjected to 9-HOT or bacterial inoculation) did not show any apparent variation in infected *noxy2* leaves with respect to control plants. These analyses suggest a positive role of NOXY2 in basal defense of plants, likely through the regulation of signals required for the full expression of resistance against biotrophic bacteria. Moreover, our results suggest the participation of NOXY2 and of the 9-LOX pathways in signaling defense responses to limit pathogen growth.

Different Signaling Pathways Involved in the Action of Oxylipins

Given that *noxy* mutants failed to form root waves when grown on 9-HOT, the set of oxylipins used in this study in wild-type *Arabidopsis* (see Supplemental Figure 1 online) was used on the *noxy2* plants to obtain information about the signaling pathways involved. Additionally, the JA-insensitive *coi1-1* mutant (Xie et al., 1998) was included in these analyses (Figure 9). Results from these studies showed that the *noxy2* plants were insensitive to the 10 waving-inducing oxylipins, whereas the *coi1-1* mutant responded as wild-type plants to all of these molecules. On the other hand, the *noxy2* mutant responded similarly to wild-type plants to the six root growth–arresting oxylipins. As expected, the *coi1-1* mutants were insensitive to the presence of the two cyclic oxylipins used, JA and its precursor OPDA. However, a root growth–arresting phenotype similar to that observed with wild-type plants was induced by 9-oxo-C_9, traumatin, colnelenic acid, or colneleic acid in *coi1-1* mutants. Results in Figure 9 show the responses of wild-type plants, *noxy2*, and *coi1-1* mutants to

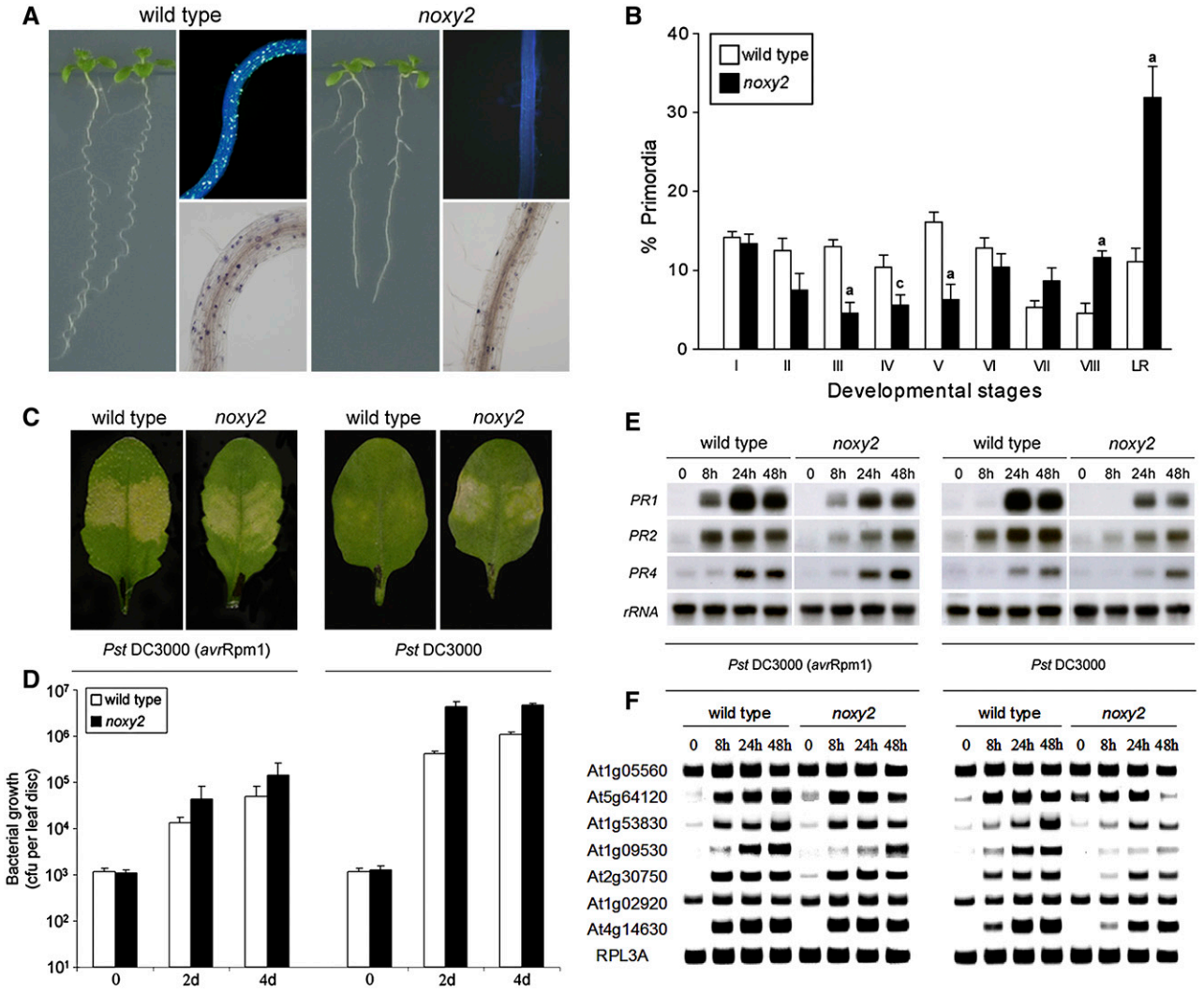


Figure 8. Characterization of the 9-HOT–Insensitive Mutant *noxy2*.

(A) Wild-type and *noxy2* seedlings grown in 9-HOT–containing medium. Shown are transmitted light visualization of seedlings, fluorescence visualization of roots stained with aniline blue, and transmitted light visualization of roots stained with NBT.

(B) Number of LRP in roots of wild-type plants and *noxy-2* mutants grown on MS medium. Means and SE of measurements of 20 seedlings are shown. Letters above the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (*t* test; $P < 0.001$ [a] and $0.001 < P < 0.05$ [c]).

(C) Lesions developed in leaves of wild-type and *noxy2* plants after bacterial inoculation. Shown are representative examples of symptoms developed at 48 h after infiltration of a suspension of *Pst* DC3000 *avrRpm1* (10^6 cfu/mL) or *Pst* DC3000 (10^5 cfu/mL).

(D) Bacterial growth in wild-type and *noxy2* plants. Means and SE obtained in three independent experiments are shown.

(E) Analyses of defense gene expression in wild-type and *noxy2* plants at different intervals after bacterial inoculation. Blots were hybridized to riboprobes for genes encoding *PR1*, *PR2*, and *PR4*. Hybridization against an 18S rRNA radioactive probe was used as a loading control. Shown are representative examples of results obtained with RNA from three independent experiments.

(F) RT-PCR analysis of 9-HOT–responsive genes in leaves of wild-type and *noxy2* plants at different intervals after bacterial inoculation. Gene At1g43170 encoding RPL3A was used to normalize transcript levels in each sample. Shown are representative examples of results obtained with RNA from three independent experiments.

treatment with 9-HOT, 9-oxo-C_9, and JA. Also, a scheme of the synthetic pathways leading to active oxylipins is shown. This suggests that the morphological changes that are induced by the three sets of oxylipins are activated through three distinct signaling cascades.

DISCUSSION

The plant oxylipin metabolome constitutes a large number of structurally diverse compounds formed by the oxygenation of fatty acids. The importance of oxylipins such as JA and its derivatives in physiological and pathological processes in plants

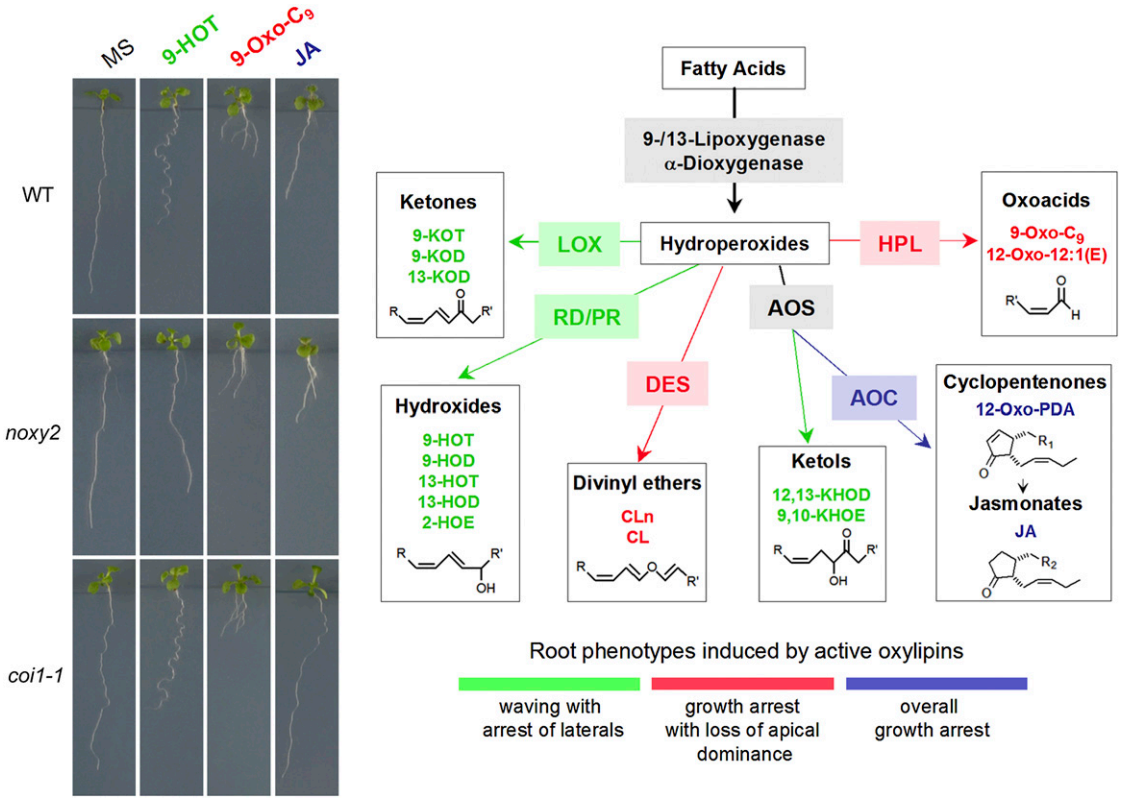


Figure 9. Root Phenotypes of Wild-Type, *noxy2*, and *coi1-1* Plants Responding to Oxylipins.

Ten-day-old seedlings grown on MS medium and in the presence of 9-HOT (25 μM), 9-oxo-C_9 (15 μM), or JA (10 μM). The scheme at right shows the branches leading to the synthesis of active oxylipins and the phenotypes induced. AOC, allene oxide cyclase; AOS, allene oxide synthase; DES, divinyl ether synthase; HPL, hydroperoxide lyase; LOX, lipoxygenase; PR, peroxygenase; RD, reductase.

is being gradually revealed. Here, we have studied the involvement of oxylipins in root growth and development, an area that to date has received limited attention. Three kinds of phenotypic alterations were observed in *Arabidopsis* germinating in the presence of oxylipins, and structure–activity relationships were readily apparent. Thus, waving of roots accompanied by inhibition of the growth of lateral roots was induced by hydroxy, keto, and keto-hydroxy oxylipins, whereas root growth arrest with a loss of apical dominance was seen in the presence of divinyl ethers and short-chain ω-oxoacids. Inhibition of overall root elongation was induced by the cyclic oxylipins JA and OPDA. Consequently, our results suggest that the metabolism of LOX-generated hydroperoxides by different secondary enzymatic branches results in oxylipins having distinct actions on roots.

Special attention was devoted to the phenotype showing root waving and inhibition of lateral roots. Of 44 oxylipins tested, the 9-LOX derivative 9-HOT was the most potent inducer of this phenotype (Figure 1; see Supplemental Figure 2 online). Interestingly, the formation of lateral roots was blocked at stage V, whereas a minor alteration was observed at the early stages of development (Figure 2), indicating that 9-HOT exerted its effect on the growth of existing lateral roots but not on their initiation. *LOX1* and *LOX5* genes encoding 9-LOXs are expressed in the LRP (Figure 3), and 9-LOX activity was found in roots of wild-type seedlings (see Supplemental Figure 3 online). Moreover, seedlings of insertion mutants lacking LOX1 or LOX5 activity as well as the *noxy2* mutant insensitive to 9-HOT all displayed an increased emergence of lateral roots (Figures 4 and 8). Together, these findings strongly indicate that 9-HOT generated locally by 9-LOX activity is causally involved in the regulation of lateral root growth, although further studies are needed to conclusively identify the endogenous 9-LOX–derived mediator.

It is known that auxins are required to initiate the formation of lateral roots as well as to continue further development to reach the postemergence stage (Celenza et al., 1995). Also, abscisic acid has been shown to regulate an auxin-independent point in lateral root development controlling meristem activation (De Smet et al., 2003). Results from microarray data (from this study and from available microarray databases [GENEVESTIGATOR; http://www.genevestigator.ethz.ch/]) suggest that 9-HOT might interfere with lateral root emergence by affecting abscisic acid signaling. Thus, whereas a low proportion (<5%) of the genes changing their expression in 9-HOT–treated roots are regulated by auxins, 30% of the root genes upregulated by 9-HOT are induced by abscisic acid (see Supplemental Table 3 online).

Histochemical characterization of the changes induced by 9-HOT treatment indicated that this oxylipin modulates root development through the modification of the cell wall. This

conclusion was based on the finding that treatment with 9-HOT led to the formation of polysaccharide deposits, composed of callose and pectin, and to the production of ROS, namely superoxide ion. Callose (β-1,3-glucan) and pectin (complex galacturonans) are components of the cell wall, and their deposition and subsequent modification allow wild-type growth and development (Micheli, 2001; Enns et al., 2005). The production of ROS also plays a developmental role by modifying cross-linking in the cell wall, causing the scission of hemicellulose molecules and the activation of calcium channels required during growth (Schopfer, 2001; Foreman et al., 2003; Carol et al., 2005). Further evidence to support the role of 9-HOT in the modification of the cell wall comes from the changes in gene expression observed in 9-HOT–treated roots. 9-HOT treatment causes transcriptional changes of genes that encode cell wall–modifying products such as pectinesterase, Hyp-rich protein, UDP-glucose transferase, and annexin, of which the latter two have been proposed to form a complex with callose synthase (Cassab, 1998; Micheli, 2001; Verma and Hong, 2001) as well as of genes that are likely to affect the level of ROS, such as peroxidase, annexin, germin-like protein (homologous with oxalate oxidase), and glucosyl oxidase (a flavin adenine dinucleotide binding domain–containing protein) (Gidrol et al., 1996; Custers et al., 2004; Tamás et al., 2005; Rouet et al., 2006).

The molecular events that are controlled by 9-HOT during development are remarkably similar to those that occur in plants as part of the active defense to control pathogen infection (Dangl and Jones, 2001). The deposition of callose as focal deposits was traditionally regarded as a barrier that reinforces the cell wall (Smart et al., 1986), although recent studies suggest that it might play a signaling role to regulate the salicylic acid pathway (Jacobs et al., 2003; Nishimura et al., 2003). As with callose, the relevance of pectin in plant defense has been highlighted by recent results showing that its alteration in the *pmr5* mutant confers resistance to certain biotrophic fungi (Vogel et al., 2004). Moreover, production of ROS is a nearly ubiquitous response to pathogen invasion, causing the reinforcement of the cell wall through the immobilization of proteins and also acting as signaling molecules to activate further defenses (Bradley et al., 1992; Lamb and Dixon, 1997; Govrin and Levine, 2000).

The finding that treatment of leaves with 9-HOT provoked an accumulation of callose as well as the production of ROS (Figure 6) is in agreement with a role of this oxylipin in plant defense. Further support for such involvement was provided by the observation that a subset of the 9-HOT root-responsive genes show enhanced expression both in leaves treated with 9-HOT and in leaves inoculated with *Pseudomonas* (Figure 7C). Additional support came from studies of the 9-HOT–insensitive *noxy2* mutant that showed increased bacterial growth and reduced activation of 9-HOT–responsive and salicylic acid–inducible genes than in wild-type plants, indicating that NOXY2 is required for full activation of resistance and that 9-HOT might be involved in this response (Figure 8). Although these findings strongly indicated that the 9-LOX oxylipin pathway plays a role in defense against pathogens, definite experimental evidence for this is still lacking. Because of the functional redundancy of multiple LOX genes and of interactions between the LOX and α-DOX pathways (Hamberg et al., 2003), studies of the role of the 9-LOX pathway in plant defense will required double or multiple mutant LOX/DOX plants rather than mutants of single enzymes. This work is under way in our laboratory.

The fact that the *noxy2* mutant was defective in both plant defense and root development indicates that both processes share common signaling components. As discussed above, the nature of the changes activated by 9-HOT in both situations suggests that the participation of this oxylipin, and thus of the 9-LOX pathway, operates mainly through the modification of the cell wall. In addition, as both cell wall components and ROS are known to signal stress responses in plants (Grant and Loake, 2000; Ellis et al., 2002; Nishimura et al., 2003), it is possible that the changes in these cellular products contribute to generate other signal molecules acting as mediators of the responses activated by 9-HOT. Further examination will be required to ascertain this point; however, results shown here suggest that the modifications effected through participation of the 9-LOX and NOXY2 proteins might help to restrain pathogen invasion and to facilitate the proper emergence of lateral roots through the cell layers of the parent root.

Finally, studies with 9-HOT–insensitive (*noxy2*) and JA-insensitive (*coi1-1*) mutants revealed that the signaling mechanisms that mediate the response to 9-HOT (as well as to the additionally identified root-waving–inducing oxylipins) are activated through a JA-independent pathway. Furthermore, we have found that this pathway differs from that used by oxoacids (9-oxo-C_9 and 12-oxo-12:1(E) and divinyl ethers [colnelenic acid and colneleic acid]) to arrest root growth and the loss of apical dominance, and that the later response is also activated independently of JA. These results indicate that the functional specialization of the different oxylipins has been accompanied by the diversification of their signaling pathways. On the other hand, the insensitivity of *noxy2* to all waving-inducing oxylipins identified (produced through 13-LOX, 9-LOX, and α-DOX activities) might reflect a partial functional redundancy of the different oxylipin biosynthetic pathways.

The results of this study indicate that, in addition to JA, other oxylipins contribute to the adaptation of plants to their environment. This would include defense against pathogens and the modulation of root architecture, an important response for the accommodation of plants to different growth situations. Also, the modification of root architecture could form part of the defense mechanisms of plants to the attack of root pathogens. In this context, the complexity of the oxylipin signaling network might enhance the adaptative flexibility of plants facing a multitude of biotic and abiotic stresses.

METHODS

Plant Material, Mutant Characterization, and Growth Conditions

Arabidopsis thaliana wild type, transgenic lines, and *coi1-1* mutants used in this study were derived from ecotype Columbia. Insertion mutants used were identified using the SIGnAL T-DNA Express *Arabidopsis* gene mapping tool (http://signal.salk.edu/). SALK lines SALK_059431(*lox1-1*), SALK_000058 (*lox1-2*), SALK_044826 (*lox5-1*), and SALK_038475 (*lox5-2*) were distributed by the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). Homozygous insertion mutants were identified

by PCR using T-DNA and gene-specific primer sets as described on the T-DNA Express homepage. To evaluate whether the isolated mutants were mRNA null mutants, semiquantitative RT-PCR was performed using gene-specific primers (sequences of primer sets used in *lox1* and *lox5* mutants are shown in Supplemental Table 2 online).

Sterilized seeds were vernalized for 3 d at 4°C and grown in vertically oriented square Petri dishes (120 mm × 120 mm; Deltalab) containing 1× MS medium, pH 6.0, 1.5% (w/v) sucrose, and 1.5% (w/v) agar (Bacto Agar; Becton-Dickinson). Metacrylate platforms designed to guarantee verticality were used to hold the plates (see Supplemental Figure 6 online). Oxylipins were added to molten medium (50°C) at the indicated concentrations and then poured onto plates. Freshly prepared plates were always used to avoid product breakdown or instability. Phenotypes were generally observed at 10 d after seed germination. An M2 population from ethyl methanesulfonate–mutagenized seeds was obtained from Lehle Seeds and used to screen for mutants not responding to oxylipins. Growth conditions were 16 h of light, 8 h of dark, and 22°C. For pathology assays, seeds were sown on soil and vernalized for 4 d at 4°C. Ten days after germination, seedlings were transferred to individual pots and grown in chambers (22°C, 70% RH, 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent illumination) under a 14-h-light/10-h-dark photoperiod. Plants were treated and examined 4 weeks after seed germination.

Oxylipins

The oxylipins used were purchased from Larodan Fine Chemicals. The chemical and stereochemical purity of all compounds was checked by gas chromatography, straight-phase high-pressure liquid chromatography, and mass spectrometry and found to be in accord with the supplier's specifications (i.e., >97%). Large-scale preparation of 9-HOT was performed by incubating linolenic acid with tomato (*Solanum lycopersicum*) fruit LOX (Matthew et al., 1977) followed by sodium borohydride reduction and isolation by straight-phase high-pressure liquid chromatography. This provided >97% pure material (up to 1 g) in yields of ~35%. A range of concentrations, from 15 to 75 μM, was tested to evaluate the effect on root development of all available products. Lesser amounts of products were also examined if required. Optimal concentrations provoking clear and reproducible phenotypes are shown in Figure 1 and were used for further analyses.

Incubation of *Arabidopsis* Roots with Linolenic Acid

Roots (500 mg) from 10-d-old *Arabidopsis* seedlings were homogenized at 0°C in 0.1 M potassium phosphate buffer, pH 6.7 (5 mL), containing linolenic acid (300 μM). The homogenate was incubated at 23°C for 30 min and subsequently extracted with diethyl ether. The material obtained was methyl-esterified and trimethylsilylated and analyzed by GC-MS using a Hewlett-Packard model 5970 mass selective detector attached to a Hewlett-Packard model 5890 gas chromatograph. In other experiments, the incubations were interrupted after 10 min by the addition of ethanol (5 mL) containing $SnCl_2$ (100 mg). After standing for 5 min, material was isolated by solvent extraction, derivatized, and analyzed by GC-MS as described above.

Histology and Histochemistry

Clarification of roots for counting root primordia was performed as described by Malamy and Benfey (1997). Primordia from ~20 seedlings were counted in each experiment for each of the plants examined. Reported data are means and SE of the results obtained in at least three independent experiments. Data was statistically analyzed by *t* test ($P < 0.001$ [a], $0.001 < P < 0.01$ [b], and $0.001 < P < 0.05$ [c]) using the GraphPad Prism version 4 computer program. For the detection of callose, plant tissues were stained with a 0.1-mg/mL solution of aniline blue fluorochrome (Sirofluor; Biosupplies) during 30 min in the dark. Samples were washed, mounted in 50% glycerol on glass microscope slides, and examined with a Leica DMR fluorescence microscope. For transverse sections, samples were fixed and embedded in Historesin according to the protocol described by Di Laurenzio et al. (1996). Pectin was visualized by staining root sections with an aqueous red ruthenium solution (0.05%) for 30 min, followed by water destaining. Callose was visualized by aniline blue staining or by incubation with a 200:1 dilution of anti-callose antibodies in PBS (Biosupplies). Nitroblue tetrazolium (Roche Diagnostics) was used to stain for the site of superoxide production, as described by Carol et al. (2005). Staining of callose and superoxide production in leaves was performed as described for roots. Leaves of 4-week-old plants were vacuum-infiltrated with the appropriate solution (water, 50 μM 9-HOT, or 50 μM linolenic acid), excised from the plants, and stained at different times after infiltration. Leaves were destained by washing in an ethanol solution before visualization.

Construction of Transgenic Lines and Analyses of GUS Activity

Genomic sequences extending to ~1 kb from the translational start site of the *Arabidopsis* α-*DOX1*, α-*DOX2*, *LOX1*, and *LOX2* genes, characterized previously (Melan et al., 1993; Bell et al., 1995; De León et al., 2002; Hamberg et al., 2005), and from the four additional LOX genomic sequences, *LOX3*, *LOX4*, *LOX5*, and *LOX6*, listed in the databases were amplified by PCR from wild-type Columbia using Expand High Fidelity polymerase (Roche). Forward and reverse primers used in each case are shown in Supplemental Table 2 online. The resulting PCR fragments were inserted into the plasmid pGEM-T Easy vector system I (Promega) and sequenced to ensure correct amplification. Promoters were fused to the coding region of the *GUS* gene present in the plasmid pBI101.2, which confers resistance to kanamycin in planta, introduced into *Agrobacterium tumefaciens*, and transferred into Columbia wild-type plants. Examination of GUS activity in transgenic seedlings was performed as described by Malamy and Benfey (1997).

Plant Treatment and RNA Isolation

From microarray analyses, 6-d-old seedlings grown on MS medium were transferred to fresh plates and grown for 3 or 5 additional days in the absence or presence of 9-HOT (25 μM). Root fragments grown after transferring to fresh plates (~20 and ~30 mm from the tip at 3 and 5 d, respectively) were excised and used to compare gene expression between controls (roots grown on MS medium) and 9-HOT–treated seedlings. Root fragments from ~400 seedlings were collected for each independent sample. Total RNA was isolated from three independent biological replicates. For chemical treatment, 4-week-old *Arabidopsis* plants were vacuum-infiltrated with 50 μM 9-HOT, and leaves were excised from the plant at the times indicated. Bacterial inoculation was performed by injecting a bacterial suspension of 10^6 cfu/mL *Pseudomonas syringae* pv *tomato* DC3000 *avrRpm1* or *P. syringae* pv *tomato* DC3000 into the abaxial side of rosette leaves of 4-week-old plants. Plant tissues were immediately frozen in liquid nitrogen after harvest. Total RNA was isolated according to Logemann et al. (1987) and then using the RNeasy mini kit (Qiagen). RNA samples were examined as described below.

Microarray Analysis

Gene expression of roots grown on MS medium versus MS medium + 9-HOT was compared using Superamine Telechem slides of the Qiagen-Operon *Arabidopsis* version 3.0 obtained from David Galbraith (Arizona University). The whole-genome oligonucleotide set represents 26,173 protein-coding genes and 28,964 transcripts (Galbraith et al., 2004).

More information about printing and the oligonucleotide set can be found at http://ag.arizona.edu/microarray/. In these experiments, 6-d-old seedlings grown on MS medium were transferred to fresh plates and grown for 3 or 5 additional days in the absence or presence of 9-HOT. RNA was prepared from the root fragments grown after transferring to fresh plates (~20 and ~30 mm from the tip at 3 and 5 d, respectively) and used to compare gene expression between controls (roots grown on MS medium) and 9-HOT–treated seedlings. Three independent hybridizations were performed for each comparison using RNAs obtained from three independent experiments, and the results were examined as described below. RNA was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technology) and assessed using an Agilent 2100 bioanalyzer (Agilent Technology). One microgram of total RNA from each sample was amplified and aminoallyl-labeled using the MessageAmp II aRNA kit (Ambion) and 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (Ambion) according to the manufacturer's instructions. A quantity (7.5 μg) of aminoallyl-labeled aRNA were resuspended in 0.1 M Na_2CO_3, pH 9.0, and labeled with either Cy3 or Cy5 Mono NHS ester (Cy Dye Postlabeling Reactive Dye Pack; Amersham). Samples were purified according to the manufacturer's instructions for Megaclear (Ambion), and Cy3 and Cy5 incorporation was measured using 1 μL of the probe in the Nanodrop spectrophotometer. Hybridization and washing of microarrays are described in the Supplemental Methods online.

Microarray Data Analysis

Background correction and normalization of the expression data were performed using the LIMMA software package (Smyth and Speed, 2003). To avoid exaggerated variability of log ratios in low-intensity spots, the normexp method in LIMMA was used to adjust the local median background estimates. Log ratio values were scaled as described by Storey (2003) to have similar distribution across arrays and consistency among arrays. The rank products method was used to determine genes differentially expressed. For each chip, probes were sorted by their normalized expression ratio in two lists of ascending and descending order. Rank products were calculated for each gene according to Breitling et al. (2004) and compared with the root primordia of 5000 random permutations of the same data to assign E-values. The multiple testing problem inherent to microarray experiments was corrected using the false discovery rate (FDR): we divided the E-value of each gene by its position in the list of changed transcripts (Storey, 2003). An FDR of 5% means that only 5% or less of the genes up to this position are expected to be observed by chance (false positives), the remaining 95% being genes that are indeed significantly affected (true positives). Significantly upregulated and downregulated genes obtained in *Arabidopsis* roots after 3 and 5 d of 9-HOT treatment (at FDR of 5%), represented in red and green, are listed in Supplemental Table 1 online in ascending order of FDR. Additionally, fold change and M values, representing differential expression ratios and their logarithmic forms, respectively, are listed for each gene. Hierarchical clustering of both significantly upregulated and downregulated genes was performed using The Institute for Genomic Research Multiexperiment Viewer version 3.0.3 using Euclidean distance and the average linkage clustering (Saeed et al., 2003). Annotation of *Arabidopsis* genes based on the probe set identifiers was obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org).

Analyses of Gene Expression

RT-PCR was performed with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the Titan One Tube RT-PCR system (Roche Applied Science) as specified by the manufacturer. Total RNA was treated with DNase TURBO DNA-free (Ambion) to remove contaminating DNA. A quantity (100 ng) of this RNA was used in each one-step RT-PCR procedure. Primers used and the lengths of amplification products are described in Supplemental Table 2 online. Gene At1g43170 encoding RPL3A was used as an internal standard. For RNA gel blots, RNA (5 μg/lane) was analyzed on agarose-formaldehyde gels, transferred to Hybond N membranes, and hybridized to single-stranded riboprobes according to standard procedures (Sambrook et al., 1989). Radioactive probes were prepared for *PR1*, *PR2* (Uknes et al., 1992), and *PR4* (EST clone 119C17, obtained from the Nottingham Arabidopsis Stock Centre). The amount of loaded RNA was verified by the addition of ethidium bromide to the samples and photography under UV light after electrophoresis, followed by hybridization to 18S rRNA (Ruiz-García et al., 1997). Blots shown are representative examples of the results obtained in three independent experiments.

Mutant Isolation

A screen of ~20,000 M2 seeds from an ethyl methanesulfonate–mutagenized population was performed. Seeds were grown on vertical MS plates for 4 d and then transferred to 9-HOT (25 μM)–containing MS plates. Seedlings failing to show a root-waving phenotype in the presence of 9-HOT were selected as putative mutants and were designated *noxy*. Progeny was retested for the same phenotype and crossed to wild-type Columbia plants to perform segregation analyses. Crosses were performed for complementation analyses. For mapping purposes, *noxy* mutants were crossed to the wild type of the C24 ecotype and F2 mutants were selected.

Analyses of Symptom Development and Bacterial in Vivo Growth Curves

Bacterial symptoms in the wild type and *noxy2* mutants were visually examined after infecting leaves of 4-week-old plants with a suspension of 10^5 or 10^6 cfu/mL *Pst* DC3000 *avrRpm1* or *Pst* DC3000. For each genotype, a minimum of 20 plants was examined in three independent experiments. Bacterial growth was evaluated by infecting leaves with a bacterial suspension of 10^5 cfu/mL. Discs of 0.6 cm^2 were excised from each infected leaf using a core borer, pooled in triplicate, and homogenized in sterile water using a plastic pestle. Eight replicates were used for each time interval examined. Bacterial populations were determined by plating appropriate dilutions from each sample in King's medium. Reported data are means and SE of the values obtained in three independent experiments.

Accession Numbers

The accession numbers for the genes discussed in this article are as follows: α*DOX1* (At3g01420), α*DOX2* (At1g73680), *LOX1* (At1g55020), *LOX2* (At3g45140), *LOX3* (At1g17420), *LOX4* (At1g72520), *LOX5* (At3g22400), *LOX6* (At1g67560), *PR1* (At2g14610), *PR2* (At3g57260), *PR4* (At3g04720), and *RPL3A* (At1g43170). Arabidopsis Genome Initiative numbers for differentially expressed genes are given in Figure 7B and in Supplemental Table 1 online. Microarray data from this article have been deposited with the ArrayExpress data library (http://www.ebi.ac.uk/arrayexpress/) under accession number E-ATMX-14.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Genes Differentially Expressed by 9-HOT Treatment in Roots.

Supplemental Table 2. Sets of Primers Used to Examine Gene Expression and to Amplify Promoters of α-*DOX* and *LOX* Genes.

Supplemental Table 3. Genes Upregulated in Roots of Control Wild-Type Plants Treated with 9-HOT (Genes Shown in Boldface Were

Found to be Induced by Abscisic Acid [Fold Change > 2] in Available Microarray Databases).

Supplemental Figure 1. Major Pathways in the Oxygenation of Fatty Acids.

Supplemental Figure 2. Dose-Dependent Response of the 9-HOT–Induced Root Waving.

Supplemental Figure 3. Oxygenation of Linolenic Acid in Roots of *Arabidopsis* Plants.

Supplemental Figure 4. Analyses of LRP in Columbia Wild-Type Plants and in *LOX1* and *LOX5* Mutants.

Supplemental Figure 5. Histological Examination of 9-HOT–Induced Waved Roots.

Supplemental Figure 6. Growth of Seedlings on Vertically Oriented Square Plates.

Supplemental Methods. Microarray Hybridization.

ACKNOWLEDGMENTS

We thank G. Bannenberg, J. Paz-Ares, and R. Solano for critical reading of the manuscript; A.P. Calvo, M. Pernas, and S. Takeda for help with histochemical analyses; I. Poveda for expert photography; R. Piqueras and M. Peinado for help with in vitro plant growth; and G. Hamberg for assistance during the preparation of the oxylipins used. The T-DNA insertion lines used in these studies were from the SALK collection and were obtained from the Nottingham Arabidopsis Stock Centre. Microarray analyses were performed at the genomic facilities of the Centro Nacional de Biotecnología (http://www.cnb.uam.es/~genomica/). This work was supported by Grant BIO2003-02338 to C.C. from the Ministry of Education and Science, by Grant GEN2003-20218 to C.C. from the Trilateral Project, by Grant 2001-2553 to M.H. from the Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning, and by Grant QLK5-CT-2001-02445 from the European Union. T.V. and J.V. were supported by fellowships from the Ministry of Education and Science.

Received July 24, 2006; revised January 18, 2007; accepted February 21, 2007; published March 16, 2007.

REFERENCES

Alméras, E., Stolz, S., Vollenweider, S., Reymond, P., Mène-Saffrané, L., and Farmer, E.E. (2003). Reactive electrophile species activate defense gene expression in *Arabidopsis*. Plant J. **34:** 205–216.

Armengaud, P., Breitling, R., and Amtmann, A. (2004). The potassium-dependent transcriptome of Arabidopsis reveals a prominent role of jasmonic acid in nutrient signaling. Plant Physiol. **136:** 2556–2576.

Bate, N.J., and Rothstein, S.J. (1998). C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. Plant J. **16:** 561–569.

Bell, E., Creelman, R.A., and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **92:** 8675–8679.

Blée, E. (2002). Impact of phyto-oxylipins in plant defense. Trends Plant Sci. **7:** 315–321.

Bradley, D.J., Kjellbom, P., and Lamb, C.J. (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defense response. Cell **70:** 21–30.

Breitling, R., Armengaud, P., Amtmann, A., and Herzyk, P. (2004). Rank products: A simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett. **573:** 83–92.

Carol, R.J., Takeda, S., Linstead, P., Durrant, M.C., Kakesova, H., Derbyshire, P., Drea, S., Zarsky, V., and Dolan, L. (2005). A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. Nature **438:** 1013–1016.

Cassab, G.I. (1998). Plant cell wall proteins. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49:** 281–309.

Celenza, J.L., Jr., Grisafi, P.L., and Fink, G.R. (1995). A pathway for lateral root formation in *Arabidopsis thaliana*. Genes Dev. **9:** 2131–2142.

Creelman, R.A., and Mullet, J.E. (1995). Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc. Natl. Acad. Sci. USA **92:** 4114–4119.

Creelman, R.A., and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48:** 355–381.

Croft, K., Jüttner, F., and Slusarenko, A.J. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiol. **101:** 13–24.

Custers, J.H., Harrison, S.J., Sela-Buurlage, M.B., van Deventer, E., Lageweg, W., Howe, P.W., van der Meijs, P.J., Ponstein, A.S., Simons, B.H., Melchers, L.S., and Stuiver, M.H. (2004). Isolation and characterisation of a class of carbohydrate oxidases from higher plants, with a role in active defence. Plant J. **39:** 147–160.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature **411:** 826–833.

De Smet, I., Signora, L., Beeckman, T., Inzé, D., Foyer, C.H., and Zhang, H. (2003). An abscisic acid-sensitive checkpoint in lateral root development of *Arabidopsis*. Plant J. **33:** 543–555.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell **86:** 423–433.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G. (2002). The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. Plant Cell **14:** 1557–1566.

Enns, L.C., Kanaoka, M.M., Torii, K.U., Comai, L., Okada, K., and Cleland, R.E. (2005). Two callose synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen development and in fertility. Plant Mol. Biol. **58:** 333–349.

Farmer, E.E., Alméras, E., and Krishnamurthy, V. (2003). Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr. Opin. Plant Biol. **6:** 372–378.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., Davies, J.M., and Dolan, L. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422:** 442–446.

Galbraith, D.W., Elumalai, R., and Gong, F.C. (2004). Integrative flow cytometric and microarray approaches for use in transcriptional profiling. Methods Mol. Biol. **263:** 259–280.

Gidrol, X., Sabelli, P.A., Fern, Y.S., and Kush, A.K. (1996). Annexin-like protein from *Arabidopsis thaliana* rescues Δ*oxyR* mutant of *Escherichia coli* from H_2O_2 stress. Proc. Natl. Acad. Sci. USA **93:** 11268–11273.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43:** 205–227.

Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. **10:** 751–757.

Grant, J.J., and Loake, G.J. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol. **124:** 21–29.

Hamberg, M., Ponce de Leon, I., Rodriguez, M.J., and Castresana, C. (2005). α-Dioxygenases. Biochem. Biophys. Res. Commun. **338:** 169–174.

Hamberg, M., Sanz, A., Rodriguez, M.J., Calvo, A.P., and Castresana, C. (2003). Activation of the fatty acid α-dioxygenase pathway during bacterial infection of tobacco leaves. Formation of oxylipins protecting against cell death. J. Biol. Chem. **278:** 51796–51805.

Howe, G.A., and Schilmiller, A.L. (2002). Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. **5:** 230–236.

Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P., and Fincher, G.B. (2003). An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell **15:** 2503–2513.

Jalloul, A., Montillet, J.L., Assigbetsé, K., Agnel, J.P., Delannoy, E., Triantaphylidès, C., Daniel, J.F., Marmey, P., Geiger, J.P., and Nicole, M. (2002). Lipid peroxidation in cotton: *Xanthomonas* interactions and the role of lipoxygenases during the hypersensitive reaction. Plant J. **32:** 1–12.

Lamb, C., and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48:** 251–275.

Liavonchanka, A., and Feussner, I. (2006). Lipoxygenases: Occurrence, functions and catalysis. J. Plant Physiol. **163:** 348–357.

Logemann, J., Schell, J., and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. Anal. Biochem. **163:** 16–20.

Lorenzo, O., and Solano, R. (2005). Molecular players regulating the jasmonate signalling network. Curr. Opin. Plant Biol. **8:** 532–540.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development **124:** 33–44.

Matthew, J.A., Chan, H.W., and Galliard, T. (1977). A simple method for the preparation of pure 9-D-hydroperoxide of linoleic acid and methyl linoleate based on the positional specificity of lipoxygenase in tomato fruit. Lipids **12:** 324–326.

Melan, M.A., Dong, X., Endara, M.E., Davis, K.R., Ausubel, F.M., and Peterman, T.K. (1993). An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol. **101:** 441–450.

Micheli, F. (2001). Pectin methylesterases: Cell wall enzymes with important roles in plant physiology. Trends Plant Sci. **6:** 414–419.

Mueller, M.J., Mène-Saffrané, L., Grun, C., Karg, K., and Farmer, E.E. (2006). Oxylipin analysis methods. Plant J. **45:** 472–489.

Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H., and Somerville, S.C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science **301:** 969–972.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Métraux, J.P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8:** 2309–2323.

De León, I.P., Sanz, A., Hamberg, M., and Castresana, C. (2002). Involvement of the *Arabidopsis* α-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. Plant J. **29:** 61–72.

Prost, I., et al. (2005). Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol. **139:** 1902–1913.

Rancé, I., Fournier, J., and Esquerré-Tugayé, M.T. (1998). The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc. Natl. Acad. Sci. USA **95:** 6554–6559.

Rouet, M.A., Mathieu, Y., Barbier-Brygoo, H., and Laurière, C. (2006). Characterization of active oxygen-producing proteins in response to hypo-osmolarity in tobacco and *Arabidopsis* cell suspensions: Identification of a cell wall peroxidase. J. Exp. Bot. **57:** 1323–1332.

Ruiz-García, L., Madueño, F., Wilkinson, M., Haughn, G., Salinas, J., and Martínez-Zapater, J.M. (1997). Different roles of flowering-time genes in the activation of floral initiation genes in Arabidopsis. Plant Cell **9:** 1921–1934.

Rustérucci, C., Montillet, J.L., Agnel, J.P., Battesti, C., Alonso, B., Knoll, A., Bessoule, J.J., Etienne, P., Suty, L., Blein, J.P., and Triantaphylidès, C. (1999). Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. J. Biol. Chem. **274:** 36446–36455.

Saeed, A.I., et al. (2003). TM4: A free, open-source system for microarray data management and analysis. Biotechniques **34:** 374–378.

Sambrook, J., Fritisch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. (2000). The Arabidopsis *DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12:** 1041–1061.

Sanz, A., Moreno, J.I., and Castresana, C. (1998). PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. Plant Cell **10:** 1523–1537.

Schopfer, P. (2001). Hydroxyl radical-induced cell-wall loosening *in vitro* and *in vivo*: Implications for the control of elongation growth. Plant J. **28:** 679–688.

Smart, M.G., Aist, J.R., and Israel, H.W. (1986). Structure and function of wall appositions. I. General histochemistry of papillae in barley (*Hordeum vulgare*) coleoptiles attacked by *Erysiphe graminis* f. sp. *hordei*. Can. J. Bot. **64:** 793–801.

Smyth, G.K., and Speed, T. (2003). Normalization of cDNA microarray data. Methods **31:** 265–273.

Staswick, P.E., Su, W., and Howell, S.H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. USA **89:** 6837–6840.

Stelmach, B.A., Müller, A., Hennig, P., Laudert, D., Andert, L., and Weiler, E.W. (1998). Quantitation of the octadecanoid 12-oxophytodienoic acid, a signalling compound in plant mechanotransduction. Phytochemistry **47:** 539–546.

Stintzi, A., and Browse, J. (2000). The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc. Natl. Acad. Sci. USA **97:** 10625–10630.

Storey, J.D. (2003). The positive false discovery rate: A Bayesian interpretation and the q-value. Ann. Statist. **31:** 2013–2035.

Tamás, L., Budíková, S., Huttová, J., Mistrík, I., ŠimonoviŠová, M., and Široká, B. (2005). Aluminum-induced cell death of barley-root border cells is correlated with peroxidase- and oxalate oxidase-mediated hydrogen peroxide production. Plant Cell Rep. **24:** 189–194.

Turner, J.G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. Plant Cell **14** (suppl.): S153–S164.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in Arabidopsis. Plant Cell **4:** 645–656.

Verma, D.P., and Hong, Z. (2001). Plant callose synthase complexes. Plant Mol. Biol. **47:** 693–701.

Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95:** 7209–7214.

Vogel, J.P., Raab, T.K., Somerville, C.R., and Somerville, S.C. (2004). Mutations in *PMR5* result in powdery mildew resistance and altered cell wall composition. Plant J. **40:** 968–978.

Vollenweider, S., Weber, H., Stolz, S., Chételat, A., and Farmer, E.E. (2000). Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased *Arabidopsis* leaves. Plant J. **24:** 467–476.

Weber, H., Chételat, A., Caldelari, D., and Farmer, E.E. (1999). Divinyl ether fatty acid synthesis in late blight-diseased potato leaves. Plant Cell **11:** 485–493.

Weichert, H., Stenzel, I., Berndt, E., Wasternack, C., and Feussner, I. (1999). Metabolic profiling of oxylipins upon salicylate treatment in barley leaves—Preferential induction of the reductase pathway by salicylate. FEBS Lett. **464:** 133–137.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). *COI1*: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094.

Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Plant Biology

Controlling hormone signaling is a plant and pathogen challenge for growth and survival

Miguel Angel López, Gerard Bannenberg and Carmen Castresana

Plants and pathogens have continuously confronted each other during evolution in a battle for growth and survival. New advances in the field have provided fascinating insights into the mechanisms that have co-evolved to gain a competitive advantage in this battle. When plants encounter an invading pathogen, not only responses signaled by defense hormones are activated to restrict pathogen invasion, but also the modulation of additional hormone pathways is required to serve other purposes, which are equally important for plant survival, such as re-allocation of resources, control of cell death, regulation of water stress, and modification of plant architecture. Notably, pathogens can counteract both types of responses as a strategy to enhance virulence. Pathogens regulate production and signaling responses of plant hormones during infection, and also produce phytohormones themselves to modulate plant responses. These results indicate that hormone signaling is a relevant component in plant–pathogen interactions, and that the ability to dictate hormonal directionality is critical to the outcome of an interaction.

Addresses
Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, Cantoblanco, E-28049 Madrid, Spain

Corresponding author: Castresana, Carmen (ccastresana@cnb.csic.es)

Current Opinion in Plant Biology 2008, **11**:420–427

This review comes from a themed issue on
Biotic Interactions
Edited by Murray Grant and Sophien Kamoun

Available online 26th June 2008

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2008.05.002

Introduction

Plants have evolved an immune system to recognize and respond to pathogen attack. In recent years there has been much progress in understanding this plant defense strategy. Numerous components involved in pathogen perception, signal generation and transmission, and activation of defense products restricting pathogen invasion, have been identified. Compelling evidence demonstrates that hormones such as salicylic acid (SA), jasmonates (JAs) and ethylene (ET) are the primary signals inducing defense responses through recognized defense hormone signaling pathways. Significant insight has also been gained into the machinery used by pathogens to invade plant tissues, including their strategies to interfere with the activation of plant defense.

Appropriate re-allocation of limiting resources to energy-costly induced defense responses, as opposed to demands for growth and reproduction, is critical to triggering immunity in plants. Emerging data is starting to unravel the mechanisms used by plants to maintain an equilibrium between these two processes, which is fundamental for their survival and effective competition with other plant species. Recent studies demonstrate that, in addition to known defense pathways (signaled by SA, JAs and ET), oxylipins other than JA, and hormones such as brassinosteroids (BL), auxins, gibberellins (GA), cytokinins (CK), and abscisic acid (ABA), play roles in plant responses to pathogen assault. Upon microbial attack, plants modify the relative abundance of these hormones, and the expression of their responsive genes, as an instrument to activate an efficient defense response allowing plant survival. Importantly, pathogens can counteract this strategy by interfering with these plant hormonal changes and also by producing plant hormones themselves as a component of their invading strategy [1•]. Here, we outline the latest discoveries showing the involvement of multiple hormone signaling pathways in plant defense, and report the most striking illustrations of the interaction between distinct pathways and how pathogens interfere with these signaling processes to colonize plants.

Activation of immunity and interactions between defense signaling pathways

Our understanding of the plant immune system has increased enormously in the last decade. A major achievement has been the demonstration that plants use two generalized modes of recognition to sense a harmful microbe. First, perception of conserved microbial molecules (generally designated as microbe associated molecular patterns or MAMPs) by pattern recognition receptors (PRRs) which are generally localized at the cell surface [2–4]. This initial recognition induces basal resistance or MAMP-triggered immunity in non-host and host plants, and acts as a first barrier to halt pathogen infection [4,5]. Second, recognition by resistance (*R*)-gene products, largely inside the cell, of specific effector molecules delivered into the plant by pathogens that have overcome the first barrier of basal resistance. This later response is designated as effector-triggered immunity (ETI) and is generally accompanied by the hypersensitive response, a local death program that ultimately restricts pathogen invasion [6–8].

Immune responses triggered by PRRs and *R*-gene-products are very similar [9,10]. However, constitutive defense components and associated signaling events playing major roles in these two immunity barriers might differ [5,11,12]. Overall, these responses involve ion fluxes across the plasma membrane, the generation of reactive oxygen intermediates (ROI), nitric oxide (NO), deposition of callose, activation of calcium-dependent and mitogen-activated protein kinases, and transcription of numerous defense genes. Many studies demonstrate that SA, JAs and ET are the main molecules signaling the activation of defense genes [13]. The SA pathway is primarily linked to resistance to biotrophic pathogens, whereas the JA and ET pathways mediate resistance mostly to necrotrophic pathogens, indicating that activation of defense signaling pathways depends on the pathogen lifestyle and its mode of infection. Frequently, these pathways interact in an antagonistic manner [14], a circumstance that can be used by pathogens to avoid plant defenses. A paradigmatic example reflecting this situation is the production by some strains of *Pseudomonas syringae* of the phytotoxin coronatine, a JA-isoleucine (JA-Ile) analog that mimics many of the responses activated by this plant stress hormone. Studies with wild type plants and JA signaling mutants revealed that coronatine contributes to the virulence of the biotrophic bacterium *P. syringae* pv. *tomato* DC3000 by suppressing the activation of SA signaling [15,16]. Additional examples of how pathogens might interfere with defense signaling are the ET production by *Botrytis cinerea* [17] and *Ralstonia solanacearum* [18]. Results with *Ralstonia* and plant ET mutants indicate a role for ET in pathogenicity through manipulation of host ET signaling [18,19].

Plant and pathogen oxylipins modulate defense and susceptibility

JA is an essential hormone for the regulation of defense and developmental responses. It belongs to a family of active molecules, the oxylipins, that originate in the oxidation of various fatty acids usually by the activities of lipoxygenases (9-lipoxygenases and 13-lipoxygenases) and α-dioxygenases. Production of oxylipins and phytoprostanes, a group of non-enzymatically formed oxylipins [20,21], is a universal response of plants to pathogen attack. Given the importance of the 13-lipoxygenases in catalyzing the first step in the JA biosynthetic pathway, research has been largely devoted to 13-lipoxygenase expression and activity. However, in recent years interest in the role of 9-lipoxygenases has revealed their participation in plant defense and developmental responses through the activation of specific signaling pathways [22•]. In *Arabidopsis thaliana* the 9-lipoxygenase genes, *LOX1* and *LOX5*, are strongly expressed in root initials, and *lox1* and *lox5* mutants display enhanced root primordial emergence. A mutant, *noxy2*, that was defective in responding to the 9-lipoxygenase product, 9-hydroxylinolenic acid (in a root-waving assay), was found not only to display alterations in root development, but also enhanced susceptibility to incompatible and compatible strains of *P. syringae*, suggesting that a 9-lipoxygenase-derived oxylipin is both a modulator of root architecture and part of the defense mechanisms against pathogen attack [22•]. Supporting these observations, the maize 9-lipoxygenase ZmLOX3 is required for both resistance to the root-knot nematode *Meloidogyne incognita* and normal plant development [23].

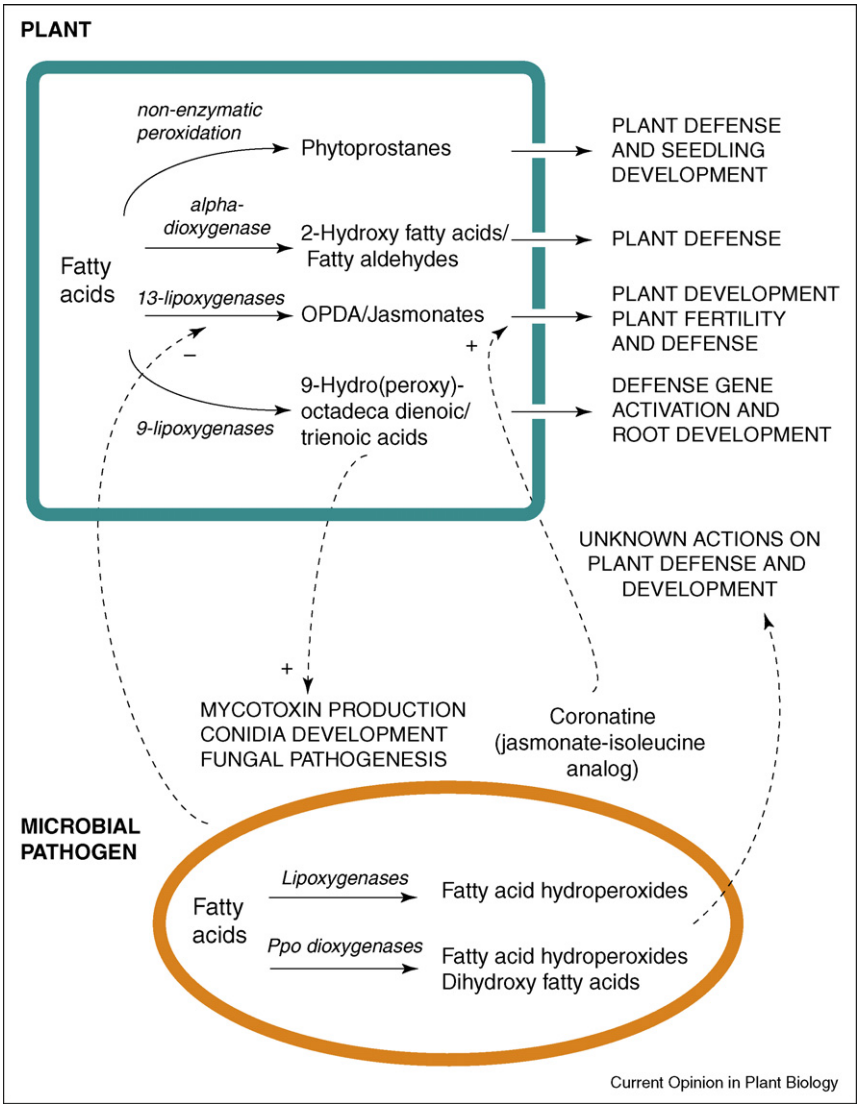
Importantly, and of significance to this review, generation of oxylipins is not restricted to plants but is widespread among pathogenic microbes [24•,25]. Oxylipin-generating oxygenases have been detected in plant pathogenic fungi including *Fusarium* spp., *Gaeumannomyces graminis*, *Laetisaria arvalis*, *Cercospora zeae-maydis*, *Ustilago maydis and Aspergillus* spp. [24•]. Molecular identification of fungal oxylipins is best characterized in *Aspergillus* spp. in which a mixture of hydroxylated oleic, linoleic and linolenic derivatives, collectively named psi factors, regulate sporulation and mycotoxin production [26]. The structural similarity of plant and fungal oxylipins, and changes in their abundance during infection, suggest that oxylipins contribute to modulate plant and pathogen interactions [27,28]. This hypothesis is supported in the interaction of *Aspergillus flavus* with peanut seeds [27], and of *F. verticillioides* and *Colletotrichum graminicola* with maize [29], where a plant 9-lipoxygenase-derived oxylipin and the 9-lipoxygenase enzymatic activity serve as fungal susceptibility factors through, respectively, stimulation of mycotoxin production and conidia formation. Established and proposed roles for plant-derived and pathogen-derived oxylipins are schematized in Figure 1.

Specific microbial compounds share significant structural similarity with oxylipins even though their biosynthesis differs from that of oxylipins, for example in the case of the *Pseudomonas* toxin coronatine, which is structurally similar to the oxylipin JA-Ile, and interferes with SA signaling [15,16]. Interestingly, coronatine can mimic JA-Ile to relieve transcriptional repression of JA-responsive genes by promoting the interaction of the COI1 F-box protein with the JA transcriptional repressors JASMONATE ZIM-domain (JAZ) proteins, which are then targeted by the SCF^{COI1}-ubiquitin ligase complex for proteasome degradation ([30,31], R Solano, personal communication). Furthermore, coronatine facilitates bacterial invasion by repressing ABA-mediated stomatal closure [32•]. Coronatine is a beautiful example of co-evolved communication between plant and pathogens highlighting emerging recognition of the importance of lipid derivatives in plant–pathogen interactions.

Changes in growth promoting hormones in plants during pathogen infection

Important growth and developmental processes are executed through signaling pathways governed by hormones

Figure 1



Plant oxylipins regulate defense and plant developmental responses. Lipoxygenases catalyze the stereospecific oxygenation of the poly-unsaturated fatty acids, linoleic acid and linolenic acid, to form hydroperoxy-containing fatty acids, which can be further converted to specific oxylipins that regulate plant development and defense. α-dioxygenase can utilize both saturated and unsaturated fatty acids to form 2-hydroperoxide-containing fatty acids that convert to 2-hydroxy fatty acids and fatty aldehydes, with regulatory functions in plant defense against pathogens and abiotic damage. Non-enzymatic oxidation pathways can generate phytoprostanes that potently activate defense mechanisms against pathogens. Pathogens can make use of plant oxylipins to enhance pathogenicity, can regulate plant oxylipin biosynthetic pathways, and a role for oxylipins generated by pathogen themselves in modulating plant host defense has been suggested. Microbial pathogens possess enzymatic pathways capable of generating specific oxylipins, including lipoxygenases, and dioxygenases identified in *Aspergillus* spp. Strains of *Pseudomonas syringae* can form a jasmonate analog, coronatine, which activates plant jasmonate signaling, downregulating salicylic acid-dependent defense pathways.

such as BL, auxins, CK and GA. In addition, there is increasing evidence that regulation of these signaling pathways helps determine the outcome of a plant–pathogen interaction.

BLs are essential hormones for plant growth and development. Genetic and molecular analyses have defined key components of the BL signaling pathway, including a cell surface leucine repeat-like kinase receptor BRI1, and a receptor kinase protein (BAK1) that associates with BRI1 to transduce the BL signal across the plasma membrane. In addition to controlling plant development, three independent studies have demonstrated a BL-independent role of BAK1 as a regulator of disease resistance [33••,34••,35••]. BAK1 was required for controlling cell death, production of ROS and restriction of biotrophic

and necrotrophic infections [33••,34••]. He *et al.* [36] demonstrated the role of BAK1 in controlling cell death during normal growth. Remarkably, BAK1 interacts with FLS2, a well-characterized pattern recognition receptor, inducing basal resistance upon interaction with the bacterial MAMP flagellin [35••]. Plants carrying *bak1* mutations show normal flagellin binding but reduced activation of flagellin-triggered immune responses. Moreover, responses to other MAMPs, such as INF1, CSP22, and EF-tu, which are not recognized by FLS2, were also diminished in *bak1* plants [34••,35••], suggesting that BAK1 interacts with other pathogen-recognition receptors to activate basal defense. That BAK1 interacts with different receptors to regulate basal defense, cell death and plant growth, demonstrates its role as a key cellular component for the activation of essential plant processes. Moreover, as gene expression profiles following application of either BLs or the active flagellin peptide flg22 showed no apparent overlap [5,9,37], BAK1 probably functions as an adaptor protein in multiple signaling pathways. BAK1 function represents a fascinating example of crosstalk between defense and hormonal pathways regulating plant development.

As with BLs, the activation of basal resistance by flagellin is also known to depend, in part, on auxin signaling as recently discussed by Robert-Seilaniantz *et al.* [1•]. Compelling experimental evidence revealed that part of the invading strategy of pathogens may be stimulation of auxin signaling, whereas suppression will favor plant resistance. Consistent with this, Chen *et al.* [38••] showed that *P. syringae* may use virulence factors such as AvrRpt2 to increase auxin levels during infection to promote disease. Increased auxin production may suppress plant defenses [38••] and change other aspects of plant physiology that could facilitate pathogen growth and invasion. As a result of this pathogen strategy, plants have evolved responses to repress auxin signaling as a component of basal defense. Downregulation of auxin responses is in part accomplished by activation of microRNAs that repress auxin signaling [39••]. Furthermore, SA treatment causes global repression of auxin-related genes, including the *TIR1* receptor, resulting in stabilization of the Aux/IAA repressor proteins and inhibition of auxin responses [40••] (Figure 2).

Knowledge of the role of GA and CK in plant–pathogen interactions (reviewed in [1•,41]) is limited. Results showing that some pathogens produce these phytohormones as part of their invading strategies [41,42] indicate that, like for other growth hormones, the GAs and CKs signaling pathways are potential pathogenicity targets. As an example, the outer capsid protein P2 of the Rice dwarf virus interacts with *ent*-kaurene oxidase, an enzyme with a key role in plant gibberellin biosynthesis. The expression of *ent*-kaurene oxidase and the level of endogenous GA_1 were lower during infection and rice plants had a dwarf phenotype, which was restored by exogenous application of GAs [43]. Similarly, CKs were implicated in the infection of the *Brassicae* family with *Plasmodiophora brassicae*, a biotrophic pathogen causing an aberrant root phenotype. In addition to producing CKs, this pathogen downregulated the degradation of plant cytokinins and induced the expression of CK receptors [44].

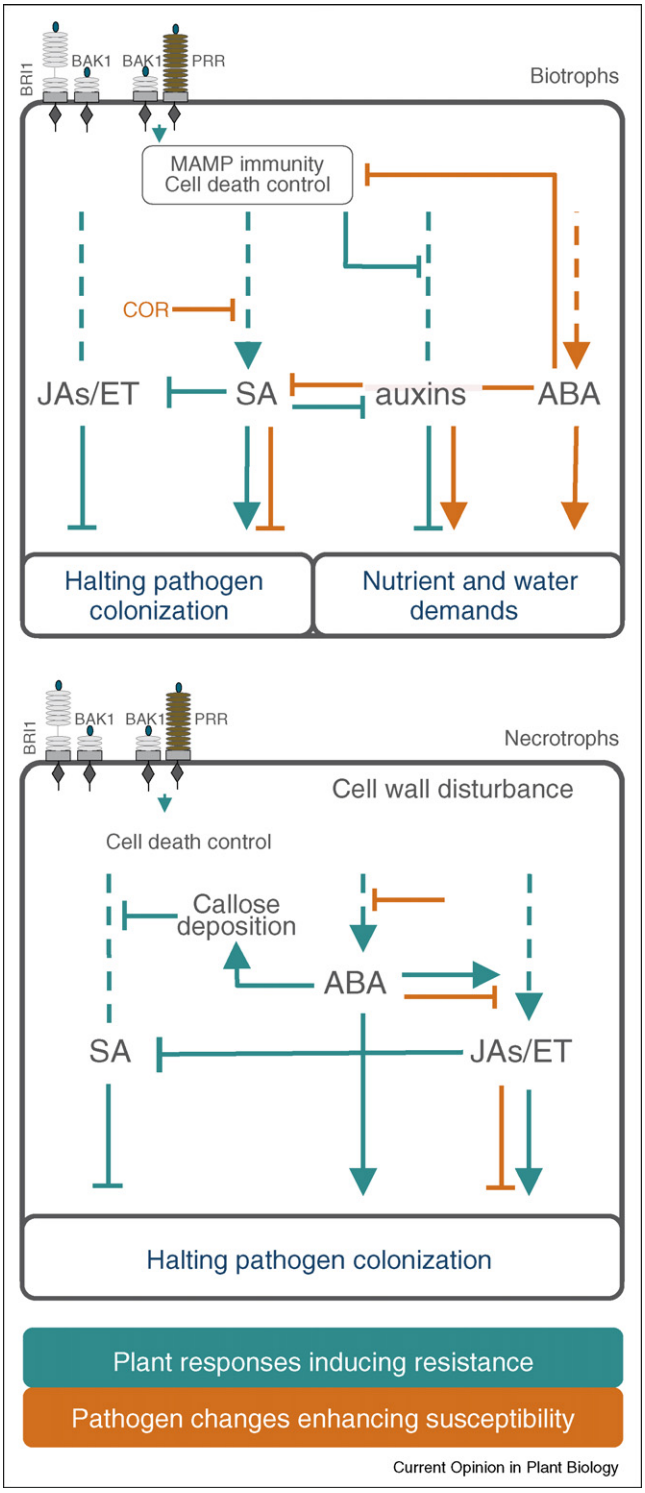
Modulation of ABA content and signaling during plant–pathogen interactions

A number of recent publications have described altered ABA levels during the interaction of plants with invading pathogens. Distinct actions of this hormone depend upon the infection stage and the specific host pathogen interaction. At a pre-invasion phase, plants enhanced resistance to application of *P. syringae* pv. *tomato* DC3000 by inducing stomatal closure and restricting pathogen entry [32•]. That the ABA-deficient mutant *aba3-1* was more susceptible to *P. syringae* pv. *tomato* DC3000 when it was sprayed-inoculated onto the leaf surface, suggests that ABA biosynthesis is required for stomatal closure in response to this bacterium [32•]. In contrast to a role in pre-apoplastic resistance, de Torres-Zabala *et al.* [45••] showed that bacterial effectors delivered into plant cells enhanced susceptibility by increasing ABA production and activating of ABA-responsive genes. In these studies ABA enhanced bacterial growth by attenuating basal defense, and ABA induction and suppression of basal defense transcripts could be mimicked by the *in planta* expression of the bacterial effector avrPtoB [45••]. ABA may thus have different actions at different infection steps, favoring resistance during pre-invasion, and susceptibility at later stages of colonization.

Increased ABA production and activation of ABA-responsive genes has also been measured in plants responding to necrotrophs. Again, opposing effects of ABA on resistance have been reported (Figure 2). ABA antagonizes resistance to *F. oxysporum* [46•], *B. cinerea* [47,48••] and *Plectosphaerella cucumerina* [49••]. Exogenous application of ABA reduced JA-activated or ET-activated transcription and pathogen resistance, whereas expression of JA-responsive genes and defense were enhanced in ABA deficient mutants. Moreover, a negative role of ABA on resistance to *B. cinerea* was supported by results showing that production of abscisic acid by *B. cinerea* itself may be involved in pathogenesis [50].

In contrast to an antagonistic effect, ABA has been recently shown to act as a positive regulator of plant defense against the necrotrophic pathogens *Phytium irregulare* and *Alternaria brassicicola* [48••,51••]. Transcriptome analyses showed the contribution of ABA as an important regulator of plant defense against the oomycete necrotrophic pathogen *P. irregulare*, resistance to which is primarily through the JA-dependent pathway. In these studies ABA levels and expression of ABA-responsive

Figure 2



Molecular actions of hormones and hormone signaling pathways in plant–pathogen interactions. The upper panel shows that resistance to biotrophs involves activation of SA-dependent defenses, to halt pathogen infection, and suppression of auxin and ABA signaling, likely to direct plant resources towards the activation of defense compounds, and to diminish the availability of nutrients and water to pathogens. Conversely, pathogens activate auxin and ABA signaling, to increase nutritional availability and water demands, and suppress SA-dependent defenses to limit the production of antimicrobial components. The lower panel shows two opposing effects of ABA in the interaction with necrotrophs: ABA enhances resistance through the positive interaction with the JA-dependent pathway, the activation of ABA-responsive defense genes and the deposition of callose. By contrast ABA enhances pathogen susceptibility through a negative interaction with the JA-dependent pathway. Also, necrotrophs might interfere with production of ABA to enhance susceptibility. BAK1, a component of the brassinosteroid signaling pathway, interacts with PRRs to activate MAMP-triggered immunity and to control cell death. Lack of BAK1 functionality enhances susceptibility to biotrophs and necrotrophs. Molecular actions of plants enhancing resistance are marked in blue. Pathogen changes promoting disease are marked in orange. Arrows represent points of entry in interacting pathways; positions of arrows within a specific pathway are indicative. SA: salicylic acid; JAs: jasmonates; ET: ethylene; ABA: abscisic acid; COR: coronatine; BRI1: brassinosteroid receptor; BAK1: brassinosteroid receptor kinase protein; PRP: pattern recognition receptors; MAMP: microbe associated molecular pattern.

genes increased after infection, and ABA-deficient or ABA-insensitive mutants were more susceptibility to *P. irregulare* and *A. brassicicola*. Interestingly, analysis with the *aba2-12* biosynthetic mutant indicated that ABA is required for JA accumulation and JA-dependent defense gene activation after *P. irregulare* infection, suggesting that ABA preceded JA action and activated defense by inducing JA biosynthesis [48••]. Corroborating a positive role of ABA in plant defense against *A. brassicicola*, Ton *et al.* [51••] showed that treatment with ABA enhanced resistance to this pathogen and that infection with an aggressive strain of *A. brassicicola* downregulated ABA accumulation to enhance pathogenicity. Accordingly, the *aba1* allelic mutant *npq2* was more susceptible to *A. brassicicola* compared to wild type plants [51••].

The action of ABA in inducing resistance is in part exerted through priming the deposition of callose [51••,52], a negative regulator of the SA-defense pathway that facilitates the activation of the JA-dependent defense pathway [53]. ABA also contributes to plant resistance by inducing expression of specific defense genes as in *Arabidopsis–Pythium irregulare* interaction [48••]. Similarly, resistance to the vascular wilting inducing bacterium *R. solanacearum*, by mutation in *CESA4/IRX5*, *CESA7/IRX3* or *CESA8/IRX1* genes, which alter secondary cell wall formation, was linked to activation of specific ABA responsive genes [49••]. Disruption of the secondary cell wall in these mutants causes structural and functional alterations in xylem vessels that affected water balance and increased ABA synthesis [54–56]. Enhanced susceptibility of ABA mutants, *abi1-1*, *abi2-1* and *aba1-6*, to *R. solanacearum* support a direct role of ABA in resistance to this pathogen [49••].

As with mechanical wounding and structural cell wall alteration [57,58], the cellular damage provoked during infection by necrotrophs might generate a water stress, in

which the production of ABA is a prominent mechanism. Necrotroph damage can thus lead to the simultaneous activation of several interacting signaling pathways that aim to control these biotic and abiotic stresses. It is possible that as infection by distinct necrotrophs might damage the cell wall differentially, the activation of defenses and their interaction with other stress signaling pathways might differ depending upon pathogen infection strategies and their endogenous suite of cell wall degrading enzymes. This may partly explain the seemingly divergent actions of ABA in plant–necrotroph interactions.

Cell wall modification is essential for normal growth and thus constitutes a potentially fundamental link between stress and growth signaling pathways. Of interest, Nemhauser *et al.* [37] found little overlap in regulation of different members within a family of cell wall-modifying enzymes (present in the *Arabidopsis* genome), with different hormones targeting specific members of the same gene family. These findings expose the complexity of hormonal signaling in controlling plant growth and highlight further complexity in the interactions of responses to biotic and abiotic stresses.

Conclusions and future prospects

Recent studies of plant–pathogen interactions have provided compelling evidence to support the importance of plant hormone signaling in determining the outcome of the interaction. By modulating hormone signaling pathways plants contribute not only directly to defense but also appear to control vital processes for resistance such as distribution of resources between defense and requirements for growth, cell death, water, and plant architecture. Pathogens on the other hand, have evolved invading strategies to access nutrient pools while limiting their exposure to antimicrobial compounds, among which the manipulation of plant hormone signaling is of major importance. Remarkably, pathogens have also developed the capacity to produce plant hormones (or analogs) to modulate plant homeostasis and defenses. These findings indicate that hormone production and signaling pathways are fundamental targets for both organisms. The action of resistance or susceptibility is likely dictated by the hormonal balance that regulates and modulates complex interacting signaling networks, whose interconnectedness are little understood. Results showing that hormones frequently act in a 'domino effect', with one hormone regulating genes involved in the metabolism of another hormone [37], allows us to envisage an intricate and mechanistically challenging panorama.

Acknowledgements

We thank J Paz-Ares and R Solano for constructive comments and stimulating discussions. This work was supported by Grant BIO2006-08581 from the Ministry of Education and Science to C.C. MAL was supported by fellowship from the Ministry of Education and Science, GB is a Ramón y Cajal fellow supported by the Spanish Ministry of Education and Science, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

References and recommended reading

Papers of particular interest, published within the period of the review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Robert-Seilaniantz A, Navarro L, Bari R, Jones JD: **Pathological hormone imbalances**. *Curr Opin Plant Biol* 2007, **10**:372-379.
The authors review the role of plant hormones in disease resistance and the interactions between hormone-mediated signaling, plant defense and pathogen virulence. Data on the production of plant hormones by pathogens is revised.

2. Gomez-Gomez L, Boller T: **FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis***. *Mol Cell* 2000, **5**:1003-1011.

3. Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G: **Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation**. *Cell* 2006, **125**:749-760.

4. Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N: **CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis***. *Proc Natl Acad Sci U S A* 2007, **104**:19613-19618.

5. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T: **Bacterial disease resistance in *Arabidopsis* through flagellin perception**. *Nature* 2004, **428**:764-767.

6. Hammond-Kosack KE, Parker JE: **Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding**. *Curr Opin Biotechnol* 2003, **14**:177-193.

7. Jones JD, Dangl JL: **The plant immune system**. *Nature* 2006, **444**:323-329.

8. Rosebrock TR, Zeng L, Brady JJ, Abramovitch RB, Xiao F, Martin GB: **A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity**. *Nature* 2007, **448**:370-374.

9. Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JD: **The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis**. *Plant Physiol* 2004, **135**:1113-1128.

10. Tsuda K, Sato M, Glazebrook J, Cohen JD, Katagiri F: **Interplay between MAMP-triggered and SA-mediated defense responses**. *Plant J* 2008, **53**:763-775.

11. Thilmony R, Underwood W, He SY: **Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7**. *Plant J* 2006, **46**:34-53.

12. Truman W, de Zabala MT, Grant M: **Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance**. *Plant J* 2006, **46**:14-33.

13. Thomma BP, Penninckx IA, Broekaert WF, Cammue BP: **The complexity of disease signaling in *Arabidopsis***. *Curr Opin Immunol* 2001, **13**:63-68.

14. Glazebrook J: **Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens**. *Annu Rev Phytopathol* 2005, **43**:205-227.

15. Brooks DM, Bender CL, Kunkel BN: **The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana***. *Mol Plant Pathol* 2005, **6**:629-639.

16. Laurie-Berry N, Joardar V, Street IH, Kunkel BN: **The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during**

infection by *Pseudomonas syringae*. *Mol Plant Microbe Interact* 2006, **19**:789-800.

17. Chague V, Elad Y, Barakat R, Tudzynski P, Sharon A: **Ethylene biosynthesis in *Botrytis cinerea***. *FEMS Microbiol Ecol* 2002, **40**:143-149.

18. Valls M, Genin S, Boucher C: **Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum***. *PLoS Pathog* 2006, **2**:e82.

19. Hirsch J, Deslandes L, Feng DX, Balangué C, Marco Y: **Delayed symptom development in *ein2-1*, an *Arabidopsis* ethylene insensitive mutant, in response to bacteria wilt caused by *Ralstonia solanacearum***. *Phytopathology* 2002, **92**:1142-1148.

20. Mueller MJ: **Archetype signals in plants: the phytoprostanes**. *Curr Opin Plant Biol* 2004, **7**:441-448.

21. Farmer EE, Davoine C: **Reactive electrophile species**. *Curr Opin Plant Biol* 2007, **10**:380-386.

22. • Vellosillo T, Martinez M, Lopez MA, Vicente J, Cascon T, Dolan L, Hamberg M, Castresana C: **Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade**. *Plant Cell* 2007, **19**:831-846.
In this study the Arabidopsis 9-Lipoxygenase pathway was found to regulate lateral root development and defense responses through a specific signaling pathway. A mutant, *noxy2*, that was defective in responding to 9-hydroxilinolenic acid (in a root-waving assay) was found not only to display alterations in root development, but also enhanced susceptibility to *Pseudomonas*.

23. Gao X, Starr J, Gobel C, Engelberth J, Feussner I, Tumlinson J, Kolomiets M: **Maize 9-lipoxygenase ZmLOX3 controls development, root-specific expression of defense genes, and resistance to root-knot nematodes**. *Mol Plant Microbe Interact* 2008, **21**:98-109.

24. • Tsitsigiannis DI, Keller NP: **Oxylipins as developmental and host-fungal communication signals**. *Trends Microbiol* 2007, **15**:109-118.
The authors review studies showing the production of oxylipins by microbial pathogens and their function as hormone-like signals to modulate development and infection.

25. Noverr MC, Erb-Downward JR, Huffnagle GB: **Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes**. *Clin Microbiol Rev* 2003, **16**:517-533.

26. Champe SP, el-Zayat AA: **Isolation of a sexual sporulation hormone from *Aspergillus nidulans***. *J Bacteriol* 1989, **171**:3982-3988.

27. Tsitsigiannis DI, Kunze S, Willis DK, Feussner I, Keller NP: ***Aspergillus* infection inhibits the expression of peanut 13S-HPODE-forming seed lipoxygenases**. *Mol Plant Microbe Interact* 2005, **18**:1081-1089.

28. Brodhagen M, Tsitsigiannis DI, Hornung E, Goebel C, Feussner I, Keller NP: **Reciprocal oxylipin-mediated cross-talk in the *Aspergillus*-seed pathosystem**. *Mol Microbiol* 2008, **67**:378-391.

29. Gao X, Shim WB, Gobel C, Kunze S, Feussner I, Meeley R, Balint-Kurti P, Kolomiets M: **Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin**. *Mol Plant Microbe Interact* 2007, **20**:922-933.

30. Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J: **JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling**. *Nature* 2007, **448**:661-665.

31. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR *et al.*: **The JAZ family of repressors is the missing link in jasmonate signalling**. *Nature* 2007, **448**:666-671.

32. • Melotto M, Underwood W, Koczan J, Nomura K, He SY: **Plant stomata function in innate immunity against bacterial invasion**. *Cell* 2006, **126**:969-980.
This paper illustrates that ABA enhances resistance by inducing stomatal closure to reduce pathogen entry, whereas coronatine interfere in this plant immune response helping to reopen plant stomatal to facilitate bacterial invasion.

33. •• Kemmerling B, Schwedt A, Rodriguez P, Mazzotta S, Frank M, Qamar SA, Mengiste T, Betsuyaku S, Parker JE, Mussig C *et al.*: **The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control**. *Curr Biol* 2007, **17**:1116-1122.
Authors report that the *Arabidopsis thaliana* brassinosteroid insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1), which operates as a coreceptor of BRI1 in BL-dependent plant development, also regulates the containment of microbial infection-induced cell death. The role of BAK1 in plant cell death control is distinct from its BL-dependent role in plant development.

34. •• Heese A, Hann DR, Gimenez-Ibanez S, Jones AM, He K, Li J, Schroeder JI, Peck SC, Rathjen JP: **The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants**. *Proc Natl Acad Sci U S A* 2007, **104**:12217-12222.
In this study BAK1, is shown to be a component of plant MAMP-triggered immunity. BAK1 interacts with FLAGELLIN SENSING 2 (FLS2), the receptor of the bacterial MAMP flagellin, to trigger immunity. BAK1, is required for full responses to multiple MAMPs in *Nicotiana benthamiana* and for restriction of bacterial and oomycete infections.

35. •• Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nurnberger T, Jones JD, Felix G, Boller T: **A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence**. *Nature* 2007, **448**:497-500.
In this study, authors show that BAK1 interacts with the flagellin receptor FLS2 to activate flagellin-triggered responses. Plans carrying *bak1* mutations show normal flagellin binding but reduced activation of MAMP-triggered immunity. BAK1 and FLS2 form a complex within the first minutes of stimulation with flagellin to initiate immunity.

36. He K, Gou X, Yuan T, Lin H, Asami T, Yoshida S, Russell SD, Li J: **BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways**. *Curr Biol* 2007, **17**:1109-1115.

37. Nemhauser JL, Hong F, Chory J: **Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses**. *Cell* 2006, **126**:467-475.

38. •• Chen Z, Agnew JL, Cohen JD, He P, Shan L, Sheen J, Kunkel BN: **Pseudomonas syringae type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology**. *Proc Natl Acad Sci U S A* 2007, **104**:20131-20136.
This paper illustrates that the *Pseudomonas syringae* type III effector AvrRpt2 modulates host auxin physiology to promote disease. Constitutive expression of AvrRpt2 in *Arabidopsis thaliana rps2* mutants (lacking a functional *RPS2* gene), increases the level of free indole acetic acid and produces phenotypes reminiscent of altered auxin physiology. Elevated levels of auxins promote PstDC3000 virulence.

39. •• Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD: **A plant miRNA contributes to antibacterial resistance by repressing auxin signaling**. *Science* 2006, **312**:436-439.
This paper demonstrates that plants have evolved responses to repress auxin signaling as a component of basal defense and that downregulation of auxin responses is in part accomplished by activation of microRNAs repressing auxin signaling.

40. •• Wang D, Pajerowska-Mukhtar K, Culler AH, Dong X: **Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway**. *Curr Biol* 2007, **17**:1784-1790.
In this paper authors show that SA causes global repression of auxin-related genes, including the *TIR1* receptor, resulting in stabilization of the Aux/IAA repressor proteins and inhibition of auxin responses.

41. Walters DR, McRoberts N: **Plants and biotrophs: a pivotal role for cytokinins?** *Trends Plant Sci* 2006, **11**:581-586.

42. Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B: **Gibberellin biosynthesis in plants and fungi: a case of convergent evolution?** *J Plant Growth Regul* 2001, **20**:319-331.

43. Zhu S, Gao F, Cao X, Chen M, Ye G, Wei C, Li Y: **The rice dwarf virus P2 protein interacts with ent-kaurene oxidases *in vivo*, leading to reduced biosynthesis of gibberellins and rice dwarf symptoms**. *Plant Physiol* 2005, **139**:1935-1945.

44. Siemens J, Keller I, Sarx J, Kunz S, Schuller A, Nagel W, Schmulling T, Parniske M, Ludwig-Muller J: **Transcriptome analysis of Arabidopsis clubroots indicate a key role for cytokinins in disease development**. *Mol Plant Microbe Interact* 2006, **19**:480-494.

45. •• de Torres-Zabala M, Truman W, Bennett MH, Lafforgue G, Mansfield JW, Rodriguez Egea P, Bogre L, Grant M: ***Pseudomonas syringae* pv. *tomato* hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease**. *EMBO J* 2007, **26**:1434-1443.
Authors demonstrate that bacterial effectors delivered into the plant cell enhance susceptibility by increasing the production of ABA and the activation of ABA responsive genes. Also, ABA is shown to enhance bacterial growth by attenuating basal defense and acting as a negative regulator of the SA plant defense pathway.

46. • Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K: **Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis***. *Plant Cell* 2004, **16**:3460-3479.
This paper illustrates a negative role of ABA in the resistance to *Fusarium oxysporum.*

47. AbuQamar S, Chen X, Dhawan R, Bluhm B, Salmeron J, Lam S, Dietrich RA, Mengiste T: **Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to Botrytis infection**. *Plant J* 2006, **48**:28-44.

48. •• Adie BA, Perez-Perez J, Perez-Perez MM, Godoy M, Sanchez-Serrano JJ, Schmelz EA, Solano R: **ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis***. *Plant Cell* 2007, **19**:1665-1681.
In this study transcriptome analyses uncovered the contribution of ABA as a positive regulator of plant defense against the oomycete pathogen *Phytium irregulare*. Authors show that ABA is required for JA production and JA-dependent defense gene activation.

49. •• Hernandez-Blanco C, Feng DX, Hu J, Sanchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sanchez-Rodriguez C *et al.*: **Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance**. *Plant Cell* 2007, **19**:890-903.
Authors show that the disruption of the secondary cell wall activate specific ABA responsive genes and confer resistance to *Plectosphaerella cucumerina* and *Ralstonia solanacearum*.

50. Siewers V, Smedsgaard J, Tudzynski P: **The P450 monooxygenase BcABA1 is essential for abscisic acid biosynthesis in *Botrytis cinerea***. *Appl Environ Microbiol* 2004, **70**:3868-3876.

51. •• Ton VFJ, van Doorn R, Jakab G, Garcia-Agustin P, Mauch-Mani B: **Interplay between JA, SA, and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola***. *Plant J* 2008, **54**:81-92.
This paper illustrates that treatment with ABA enhances resistance to *Alternaria brassicicola*, whereas pathogen infection downregulated ABA accumulation to enhance pathogenicity.

52. Ton J, Mauch-Mani B: **Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose**. *Plant J* 2004, **38**:119-130.

53. Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC: **Loss of a callose synthase results in salicylic acid-dependent disease resistance**. *Science* 2003, **301**:969-972.

54. Turner SR, Somerville CR: **Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient in cellulose deposition in the secondary cell wall**. *Plant Cell* 1997, **9**:689-701.

55. Chen Z, Hong X, Zhang H, Wang Y, Li X, Zhu JK, Gong Z: **Disruption of the cellulose synthase gene, *AtCesA8/IRX1*, enhances drought and osmotic stress tolerance in Arabidopsis**. *Plant J* 2005, **43**:273-283.

56. Brown DM, Zeef LA, Ellis J, Goodacre R, Turner SR: **Identification of novel genes in *Arabidopsis* involved in secondary cell wall formation using expression profiling and reverse genetics**. *Plant Cell* 2005, **17**:2281-2295.

57. Cheong YH, Chang HS, Gupta R, Wang X, Zhu T, Luan S: **Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis***. *Plant Physiol* 2002, **129**:661-677.

58. Huckelhoven R: **Cell wall-associated mechanisms of disease resistance and susceptibility**. *Annu Rev Phytopathol* 2007, **45**:101-127.

Functional Analysis of α-DOX2, an Active α-Dioxygenase Critical for Normal Development in Tomato Plants[1][W]

Gerard Bannenberg[2], Marta Martínez[2], María José Rodríguez[2], Miguel Angel López, Inés Ponce de León, Mats Hamberg, and Carmen Castresana*

Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain (G.B., M.M., M.J.R., M.A.L., C.C.); Departamento Biología Molecular, Instituto de Investigaciones Biológicas Clemente Estable, 11600 Montevideo, Uruguay (I.P.L.); and Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, 17177 Stockholm, Sweden (M.H.)

Plant α-dioxygenases initiate the synthesis of oxylipins by catalyzing the incorporation of molecular oxygen at the α-methylene carbon atom of fatty acids. Previously, α-DOX1 has been shown to display α-dioxygenase activity and to be implicated in plant defense. In this study, we investigated the function of a second α-dioxygenase isoform, α-DOX2, in tomato (*Solanum lycopersicum*) and Arabidopsis (*Arabidopsis thaliana*). Recombinant Slα-DOX2 and Atα-DOX2 proteins catalyzed the conversion of a wide range of fatty acids into 2(*R*)-hydroperoxy derivatives. Expression of *Slα-DOX2* and *Atα-DOX2* was found in seedlings and increased during senescence induced by detachment of leaves. In contrast, microbial infection, earlier known to increase the expression of *α-DOX1*, did not alter the expression of *Slα-DOX2* or *Atα-DOX2*. The tomato mutant *divaricata*, characterized by early dwarfing and anthocyanin accumulation, carries a mutation at the *Slα-DOX2* locus and was chosen for functional studies of α-DOX2. Transcriptional changes in such mutants showed the up-regulation of genes playing roles in lipid and phenylpropanoid metabolism, the latter being in consonance with the anthocyanin accumulation. Transgenic expression of *Atα-DOX2* and *Slα-DOX2* in *divaricata* partially complemented the compromised phenotype in mature plants and fully complemented it in seedlings, thus indicating the functional exchangeability between α-DOX2 from tomato and Arabidopsis. However, deletion of Atα-DOX2 in Arabidopsis plants did not provoke any visible phenotypic alteration indicating that the relative importance of α-DOX2 in plant physiology is species specific.

Plants have evolved elaborate signaling systems to regulate a variety of physiological responses to the environment and to facilitate intercellular cross talk in development and reproduction. Oxylipins comprise a large class of oxygenated fatty acid-derived lipid mediators that contribute to such signaling circuits (Weber, 2002; Farmer et al., 2003). A variety of functions have been ascribed to plant oxylipins, including critical roles in plant defense against microbial pathogens, as well as in reproduction and tissue development (Howe and Schilmiller, 2002; Browse, 2005; Kachroo and Kachroo 2009).

The biosynthesis of oxylipins is initiated by hydroperoxide formation catalyzed by fatty acid oxygenases, among which the 9- and 13-lipoxygenases have been studied most intensively (Shibata and Axelrod, 1995; Feussner and Wasternack, 2002). α-Dioxygenase, first encountered about 10 years ago, also catalyzes primary fatty acid oxygenation. This enzyme was first identified in *Nicotiana tabacum* plants as a pathogen-induced protein showing homology to mammalian prostaglandin endoperoxide synthases (Sanz et al., 1998). Studies of the catalytic function of the recombinant tobacco protein and of a homologous protein from Arabidopsis (*Arabidopsis thaliana*) revealed that these plant enzymes, designated as α-dioxygenase-1 or α-DOX1, catalyze the incorporation of molecular oxygen at the α-methylene carbon atom of fatty acids. The products are chemically unstable 2(*R*)-hydroperoxy fatty acids, which are either reduced to 2*R*-hydroxy fatty acid or spontaneous decarboxylated to the corresponding lower fatty aldehyde (Hamberg et al., 1999).

Expression of α-DOX1 in tobacco and Arabidopsis leaves is activated in response to bacterial inoculation and by agents that generate oxidative stress (Sanz et al., 1998; Ponce de León et al., 2002; Hamberg et al., 2003). In these responses, α-dioxygenase was proposed to play a tissue-protective role as concluded from results showing a negative correlation between the extent of α-dioxygenase activity and the level of

[1] This work was supported by the Ministry of Education and Science (Spain; grant no. BIO2006–08581 to C.C.). G.B. is a Ramón y Cajal fellow supported by the Spanish Ministry of Education and Science and the Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Spain.

[2] These authors contributed equally to the article.

* Corresponding author; e-mail ccastresana@cnb.csic.es.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Carmen Castresana (ccastresana@cnb.csic.es).

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.109.145094

tissue damage (Ponce de León et al., 2002). In addition, α-dioxygenase expression has been shown to be upregulated in response to herbivore attack (Hermsmeier et al., 2001), and a variety of different types of abiotic stresses, such as salt stress, cold, drought, and heavy metal exposure (Wei et al., 2000; Seki et al., 2002; Koeduka et al., 2005); however, the contribution of the α-DOX activity during these responses remains unknown.

Sequence analyses of reported α-dioxygenases as well as database searches reveal the presence of plant proteins that show high level of homology to the first identified α-dioxygenases, α-DOX1, in plant species such as rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), *N. tabacum*, *Nicotiana attenuata*, *Pisum sativum*, *Capsicum annuum*, *Vitis vinifera*, *Ricinus communis*, and *Populus trichocarpa* (Supplemental Fig. S1). Additionally, these analyses identify a second group of predicted α-dioxygenases, termed α-DOX2, which cluster together as a phylogenetic group distinct from the first identified α-dioxygenases (Hamberg et al., 2005; Supplemental Fig. S1). In tomato, mutations of α-DOX2, also known as FEEBLY, and *divaricata* (*div*) result in defects in plant development as well as accumulation of anthocyanins, pointing to a role of Slα-DOX2 in plant development (Stevens and Rick, 1986; van der Biezen et al., 1996). However, no information is available on the enzymatic activity of Slα-DOX2 or on any other putative α-DOX2 reported to date.

This study concerns the biochemical characterization and function of the α-DOX2 proteins from tomato (Slα-DOX2) and Arabidopsis (Atα-DOX2). We show that these two α-DOX2 enzymes are functionally interchangeable. However, in contrast to tomato in which mutation of α-DOX2 provokes large phenotypic effects, mutation of Atα-DOX2 does not have any visible phenotypic consequence.

RESULTS

α-Dioxygenase Activity of Tomato and Arabidopsis α-DOX2

Infection of High Five insect cells with recombinant baculovirus containing *Slα-DOX2* pFastBac or *Atα-DOX2* pFastBac constructs, respectively, resulted in the expression of Slα-DOX2 and Atα-DOX2 proteins with molecular masses in accordance with the predicted size (72.8 and 72.5 kD, respectively; Supplemental Fig. S2). No α-dioxygenase was detected when insect cells were infected with baculovirus prepared from empty pFastBac vector. Incubation of palmitic acid (C16:0) with Slα-DOX2-expressing cell homogenates led to the generation of the corresponding 2-hydroperoxy fatty acid as shown by the identification of the decarboxylation product pentadecanal and the reduction product 2-hydroxypalmitic acid by gas chromatography-mass spectrometry (GC-MS; Fig. 1A). In a similar fashion, Slα-DOX2 catalyzed the formation of pairs of aldehydes and 2-hydroxy acids when incubated with stearic acid (C18:0), linolenic acid (C18:3), or arachidic acid (C20:0; data not shown). Likewise, incubations of the mentioned fatty acids with Atα-DOX2-expressing cells led to the formation of 2-hydroperoxides as shown by the identification of corresponding aldehydes and 2-hydroxy acids (Fig. 2, A and C). Steric analysis of the (−)-menthoxycarbonyl derivative of 2-hydroxylinolenic acid isolated after incubation of linolenic acid with Slα-DOX2 and of 2-hydroxypalmitic acid isolated after incubation of palmitic acid with Atα-DOX2 demonstrated exclusive formation of the 2(*R*) enantiomers (Figs. 1B and 2B). These results demonstrated that both Slα-DOX2 and Atα-DOX2 catalyze stereospecific introduction of molecular oxygen at the α-carbon to produce fatty acid 2 (*R*)-hydroperoxides (Supplemental Fig. S3).

In order to determine the substrate specificity of Slα-DOX2 and Atα-DOX2, the oxygenation rates of a wide range of long chain (C14-22) and very-long-chain (C24-30) fatty acids (VLCFAs) were determined. Both enzymes oxygenated all of these fatty acids. Differences in oxygenation rates observed with Slα-DOX2 were not large (Fig. 1C). Thus, linoleic acid was oxygenated most efficiently, but only 4 times faster than its least efficient substrate, i.e. arachidic acid. Atα-DOX2 displayed a substrate preference for unsaturated fatty acids of 18 carbon chain length, i.e. oleic, linoleic, and linolenic acids, and unexpectedly also showed high activity with myristic acid (C14:0; Fig. 2D). VLCFAs ranging in chain length from 20 to 30 carbons were also efficiently oxygenated by both enzymes. These results demonstrate that the gene products of *Slα-DOX2* and *Atα-DOX2* are authentic α-dioxygenases, which can oxygenate a wide range of fatty acids.

Slα-DOX2 Activity Is Required for Normal Vegetative Growth and Fruit Development

Previously, based on allele complementation tests and on phenotypic similarities of the mutation, the α-DOX2 was shown to correspond to DIV (van der Biezen et al., 1996). Here, sequence comparison of the *Slα-DOX2* gene from both wild-type and *div* plants revealed a single nucleotide deletion in exon 9 of *Slα-DOX2* creating a stop codon at amino acid 433 of the predicted protein, thus confirming the identity of α-DOX2 and DIV (Fig. 3A). As a result of this mutation, the *div* mutant displayed significant phenotypic alterations and was selected here to further examine the function of the Slα-DOX2 protein. To this end, expression and phenotypic analyses were performed in wild-type and *div* plants. Analyses of *Slα-DOX2* expression revealed the presence of *Slα-DOX2* RNA in seedlings of wild-type plants (Fig. 3H). Expression was found preferentially in aerial tissues with the highest levels of transcript accumulation in the hypocotyls. *Slα-DOX2* expression is lower in leaves and decreased markedly as plants matured. Analyses of the two *α-DOX1*

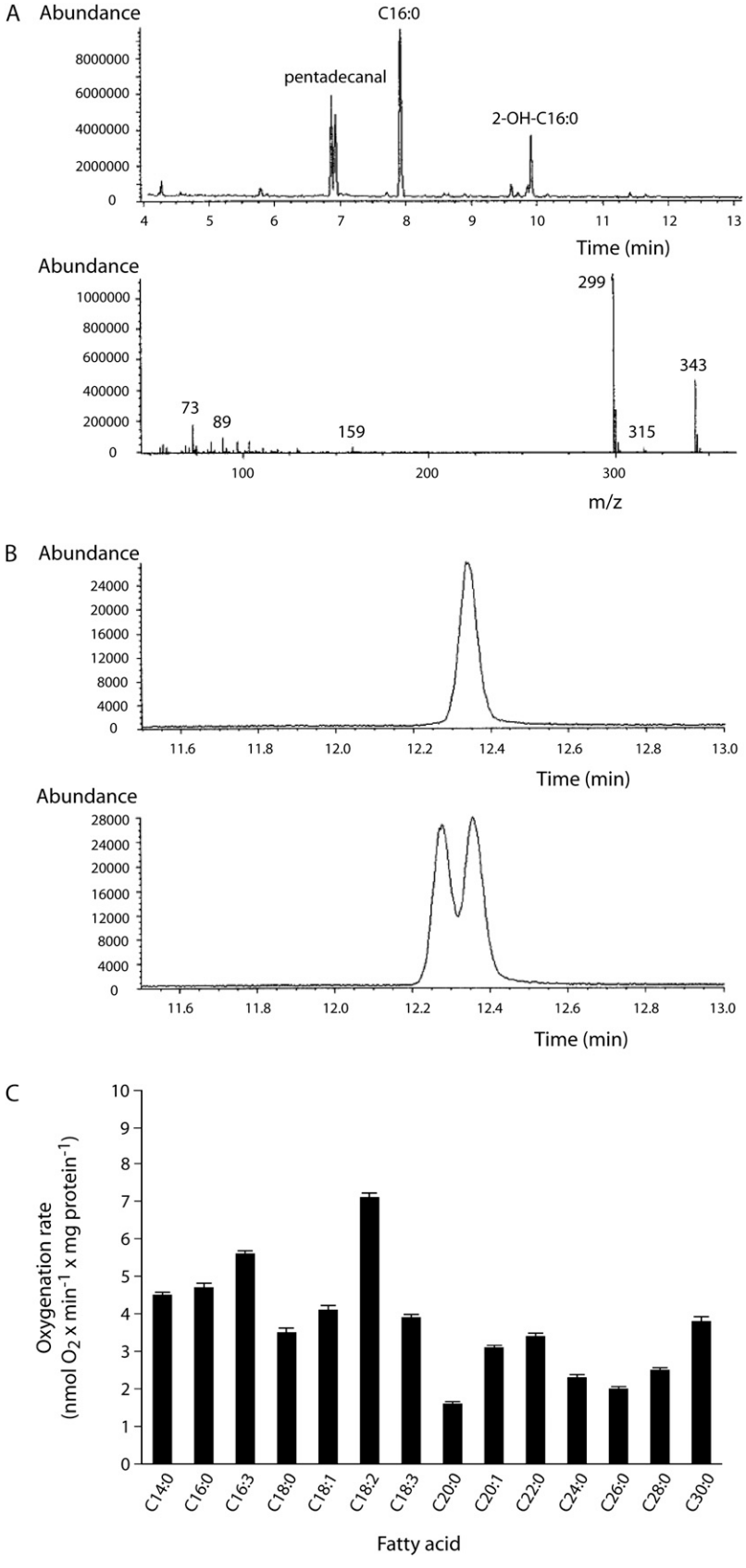
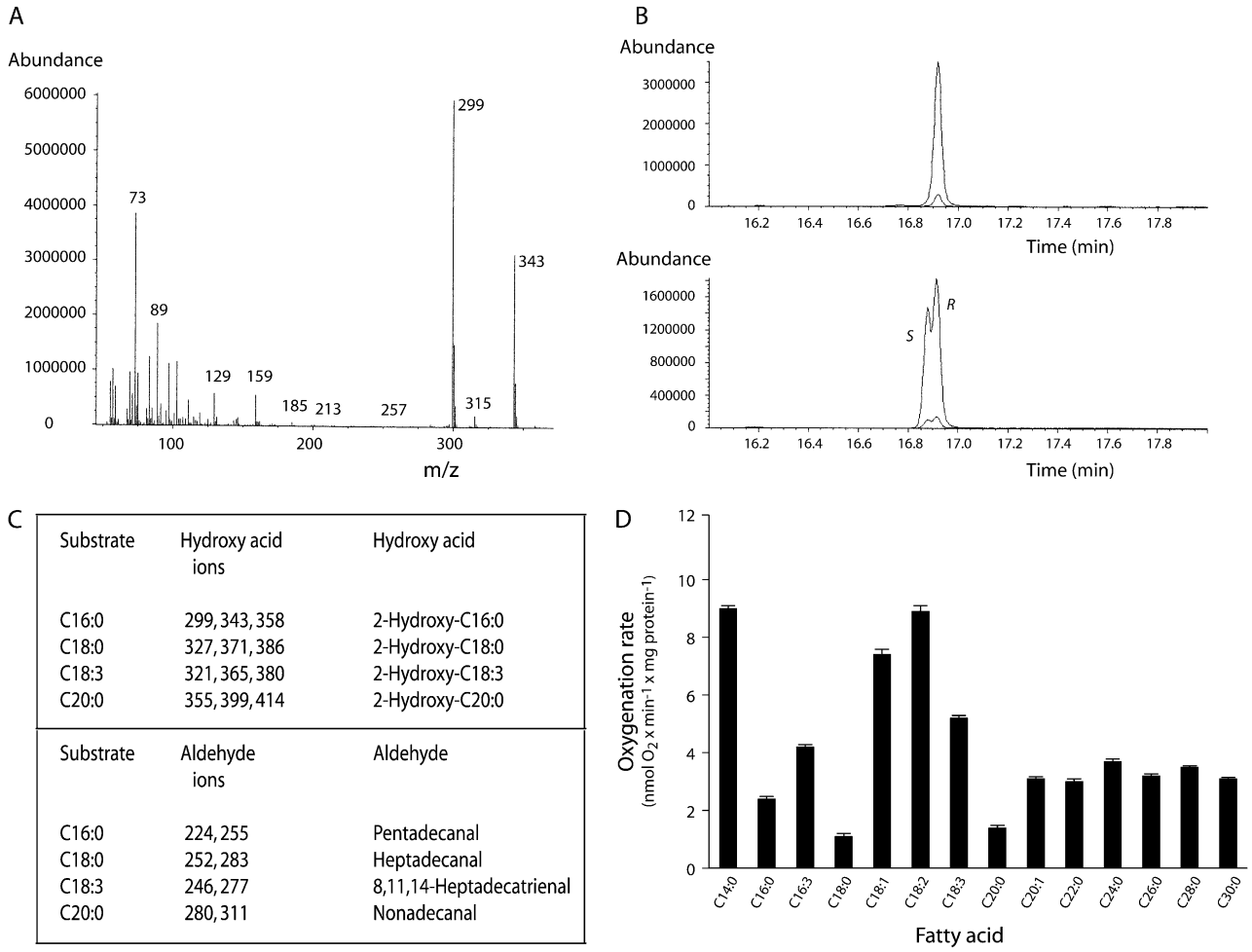


Figure 1. Determination of α-dioxygenase activity of Slα-DOX2. A, GC-MS identification of products formed by incubation of palmitic acid with Slα-DOX2-containing insect cells. Top: Peaks due to pentadecanal (*O*-methyloxime *syn/anti* isomers), palmitic acid (methyl ester; corresponding to substrate remaining not converted), and 2-hydroxypalmitic acid (methyl ester/trimethylsilyl ether derivative) were observed. The reaction products observed arouse by decarboxylation or reduction of 2-hydroperoxypalmitic acid, the primary α-DOX product. Bottom: Mass spectrum of 2-hydroxypalmitic acid (methyl ester/trimethylsilyl ether derivative). B, Steric analysis of 2-hydroxylinolenic acid as its (−)-menthoxycarbonyl/methyl ester derivative. Top: 2-Hydroxylinolenic acid prepared from an incubation of linolenic acid with Slα-DOX2. Bottom: Synthetic 2(*R*,*S*)-hydroxylinolenic acid elution order 2(*S*) followed by 2(*R*). C, Fatty acid substrate specificity of oxygenation by Slα-DOX2 (mean ± SE of n = 3–4 measurements). Enzymatic oxygenation rates were determined at 23°C after addition of approximately 100 μg total protein to 1.5 mL 0.1 M Tris, pH 7.4, containing 100 μM fatty acid substrate and 100 μM *tert*-butylhydroperoxide.



Substrate	Hydroxy acid ions	Hydroxy acid
C16:0	299, 343, 358	2-Hydroxy-C16:0
C18:0	327, 371, 386	2-Hydroxy-C18:0
C18:3	321, 365, 380	2-Hydroxy-C18:3
C20:0	355, 399, 414	2-Hydroxy-C20:0

Substrate	Aldehyde ions	Aldehyde
C16:0	224, 255	Pentadecanal
C18:0	252, 283	Heptadecanal
C18:3	246, 277	8,11,14-Heptadecatrienal
C20:0	280, 311	Nonadecanal

Figure 2. Determination of α-dioxygenase activity of Atα-DOX2. A, MS identification of 2-hydroxypalmitic acid formed by incubation of palmitic acid with a homogenate of Atα-DOX2-expressing insect cells. The methyl ester/trimethylsilyl ether derivative was used. B, Steric analysis of 2-hydroxypalmitic acid as its (−)-menthoxycarbonyl/methyl ester derivative. Top: 2-Hydroxypalmitic acid prepared from an incubation of palmitic acid with Atα-DOX2. Bottom: Synthetic 2(*R*,*S*)-hydroxypalmitic acid elution order 2(*S*) followed by 2(*R*). C, Mass-spectral ions (*m/z*) recorded on Atα-DOX2-derived 2-hydroxy fatty acids (methyl ester/trimethylsilyl ether derivatives) and fatty aldehydes (*O*-methyloxime derivatives). D, Fatty acid substrate specificity of oxygenation by Atα-DOX2 (mean ± SE of $n = 3–4$ measurements).

genes found in tomato, designated as *Slα-DOX1.1* and *Slα-DOX1.2* (Supplemental Fig. S1), revealed their expression mainly in roots, although a low level of *Slα-DOX1.2* transcript was found also in hypocotyls (Fig. 3H). The expression of *Slα-DOX2* was strongly reduced in *div* plants compared to wild-type plants.

As *α-DOX1* from different plant species has previously been shown to be induced in response to biotic stress (Sanz et al., 1998; Ponce de León et al., 2002), the expression of *Slα-DOX2* was examined in leaves from wild-type tomato infected with *Pseudomonas syringae* pv *tomato* or with the necrotrophic fungus *Botrytis cinerea*; however, these treatments did not lead to *Slα-DOX2* expression (data not shown). Only one stimulus was found to consistently activate *Slα-DOX2* expression, namely, the detachment of mature leaves, a treatment that is frequently used to induce senescence (Gepstein et al., 2003; Guo and Gan, 2005). *Slα-DOX2* mRNA expression increased in leaves 3 d after detachment and was maintained elevated up to at least 1 week after treatment (Fig. 3I). In contrast, no marked increase of *Slα-DOX2* mRNA levels was found after detachment in *div* plants (Fig. 3I).

The expression of *Slα-DOX2* in growing seedlings is in accordance with the phenotypic alterations of *div*, i.e. delayed development accompanied by strong anthocyanin accumulation in cotyledons and leaves of young plants (Fig. 3, B and C). Also, in line with the activation of *Slα-DOX2* during senescence induced by leaf detachment, a marked anthocyanin production was found in senescent leaves of *div* (Fig. 3, F and G). Inspection of mature *div* plants revealed further phenotypic alterations, including increased internodal length and reduced number of lateral shoots (Fig. 3,

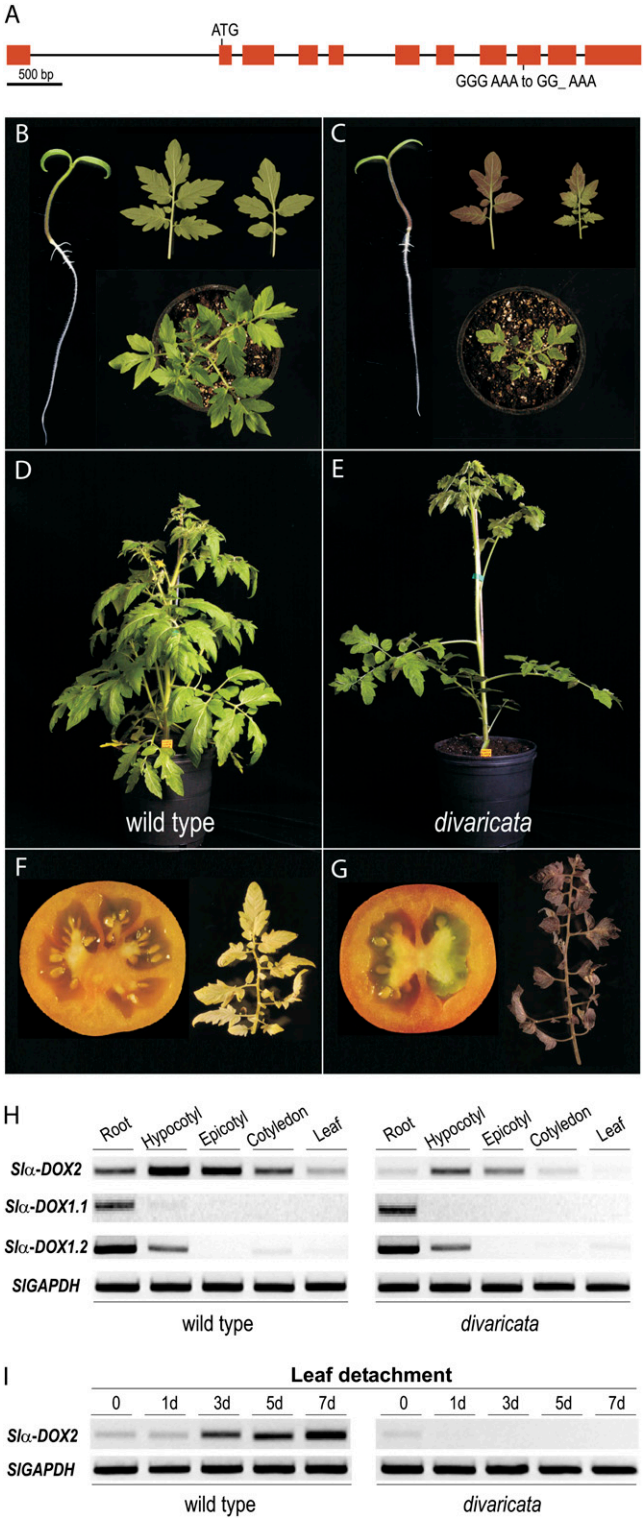


Figure 3. Vegetative growth and fruit development in wild-type and *div* tomato plants. A, Genomic structure of *Slα-DOX2* gene (GenBank accession no. FN428743), indicating the *div* mutation. B, Seedlings, young leaves, and top view of wild-type plants. C, Seedlings, young leaves, and top view of *div* plants. D, Lateral view of adult wild-type tomato plant (5 weeks old). E, Lateral view of adult *div* tomato plant (5 weeks old). F, Cross section of ripe fruit and senescent leaf of wild-type tomato plants. G, Cross section of ripe fruit and senescent leaf of *div* mutant. H, Gene expression levels of the three tomato α-dioxygenase genes *Slα-DOX2*, *Slα-DOX1.1*, and *Slα-DOX1.2* in roots, hypocotyls, epicotyls, cotyledons, and leaves of seedlings of tomato wild-type or *div* plants. I, Gene expression levels of *Slα-DOX2* during a 1-week period after detachment of young leaves of wild-type and *div* plants.

D and E), as well as a reduction in the number of locules from 3 to 10 in fruits from wild-type plants to a single symmetrical division into two locules (Fig. 3, F and G). However, no significant variations between control and mutant plants were detected in traits such as weight and size of the fruits or seed yield. Taken together, the morphological changes recapitulated in Figure 3 are consistent with a role of Slα-DOX2 at different stages of plant development. Moreover, the alterations found in mature plants suggested that *Slα-DOX2* might be expressed in additional tissues to those identified here, in which the defect of Slα-DOX2 could account for the observed phenotypic alterations.

Identification of Gene Expression Changes in *div* Plants

To gain further understanding of the function of Slα-DOX2, gene expression profiling was performed on *div* and wild-type plants. Three individual microarrays were hybridized using RNA extracted from three independent biological replicates. Complete microarrays data sets were analyzed, and a list of differentially expressed genes is shown in Supplemental Table S1. Details of computational methods to process gene expression are described in "Materials and Methods." Of over 9,200 tomato transcripts represented on the Affymetrix tomato array, 42 showed altered expression according to a statistical false discovery rate (FDR) value of 0.05. Most changes correspond to genes induced in *div* plants (69% of genes shown in red in Supplemental Table S1), while a lower proportion (31% of genes shown in green in Supplemental Table S1), including *Slα-DOX2*, decreased their expression compared to wild-type plants. In accordance with the accumulation of anthocyanins, we found that genes related to the flavonoid-phenylpropanoid pathway were among the up-regulated genes in *div* seedlings. From these, the activation of *DFR* encoding dihydroflavonol 4-reductase, an enzyme that directs anthocyanin biosynthesis by catalyzing the conversion of dihydroflavonols to leucoanthocyanidins (Lepiniec et al., 2006), was confirmed by reverse transcription (RT)-PCR (Fig. 4). In addition, a substantial percentage of the genes with increased expression in *div* (28%) encoded proteins predicted to play a role in lipid deacylation, lipid transfer, and lipid metabolism. Among these, the activation of two GDSL-motif lipase/hydrolases, one class 3 lipase, three lipid transfer proteins, and two proteins mediating the synthesis of VLCFAs (CER1 and KCS6), was confirmed by semi-quantitative RT-PCR (Fig. 4). Additional genes related to lipid modifications with a higher FDR value (be-

Figure 4. Gene expression analysis in the *div* mutant compared to wild-type tomato plants. Differentially expressed transcripts obtained from microarray analyses were examined by RT-PCR. Fold change and statistical value FDR are indicated for each probe from Affymetrix GeneChip Tomato Genome Array. Tomato *GAPDH* was used to normalize transcript levels in each sample. Gene-specific primer sets used for the evaluation of RNA are shown in Supplemental Table S2.

Fold Change	FDR (RankProd)	Affymetrix Probe ID	Description	RT-PCR (wild type / *divaricata*)
-11.63	0.000	Les.852.1.A1_at	Non-coding RNA	
+3.65	0.020	Les.3659.1.S1_at	DFR (Dihydroflavonol 4-reductase)	
+7.62	0.000	Les.1079.1.S1_at	GDSL-motif lipase/hydrolase	
+4.31	0.014	Les.4467.1.S1_at	Non-specific lipid transfer protein	
+3.93	0.015	Les.3292.2.S1_at	CER1: Aldehyde decarbonylase	
+3.79	0.016	LesAffx.55337.1.S1_at	KCS6 (CER6): Ketoacyl-CoA-synthase	
+3.76	0.017	Les.1389.1.S1_at	LTPG2: Non-specific lipid transfer protein	
+3.56	0.018	LesAffx.14185.1.S1_at	Non-specific lipid transfer protein	
+4.09	0.022	Les.4291.1.S1_at	GDSL-motif lipase/hydrolase	
+3.12	0.031	LesAffx.7472.1.S1_at	Lipase class 3 family protein	
+2.94	0.052	LesAffx.70407.1.S1_at	Lipid transfer protein-related	
+2.12	0.141	Les.4187.1.S1_at	LTPG1: Non-specific lipid transfer protein	
+2.20	0.153	LesAffx.52290.1.S1_at	GDSL-motif lipase/hydrolase	
+2.18	0.164	Les.4333.1.S1_at	Lipid transfer protein	
+2.10	0.164	Les.3668.1.S1_at	LOXA: Lipoxygenase 9-LOX	
+2.00	0.186	LesAffx.68271.1.S1_at	CYP94B1: Cytochrome P450	
+1.33	0.705	AFFX-Les-gapdh-5_at	GAPDH: glyceraldehyde-3-phosphate dehydrogenase	

tween 0.05 and 0.21) were also selected for RT-PCR examination. These included genes encoding a GDSL-motif lipase/hydrolase, three lipid transfer proteins, a lipoxygenase, and a cytochrome P450 CYP94B1 that has been implicated in the biosynthesis of suberin (Kandel et al., 2006). As shown in Figure 4, the expression of the former five first genes was confirmed by RT-PCR and found to be increased in *div*, whereas no significant change was observed for CYP94B1. Finally, a high percentage of genes with decreased expression in *div* plants with respect to controls were chloroplastic (54%). Within the down-regulated group of genes, the expression of a nuclear gene (cDNA sequence with GenBank accession no. AK247303) encoding a noncoding RNA was confirmed by RT-PCR (Fig. 4).

Taken together, the results from these studies showed that the mutation of Slα-DOX2 leads to increased anthocyanin production by a pretranslational control mechanism. Moreover, the additional identified transcriptional changes suggest that the *div* mutation would give rise to important changes in lipid composition of the plant.

Functional Analyses of Arabidopsis α-DOX2

Given that Slα-DOX2 and Atα-DOX2 cluster together within a phylogenetic group distinct from that of the first identified α-DOX1 α-dioxygenases (see Supplemental Fig. S1), we speculated that Atα-DOX2 might be a functional homolog of Slα-DOX2. To examine this possibility, we assessed whether Arabidopsis mutants defective in *Atα-DOX2* display developmental alterations as observed for *Slα-DOX2* in tomato. To this end, two Arabidopsis T-DNA insertion mutants, *α-dox2-1* and *α-dox2-2*, lacking Atα-DOX2 function (see Supplemental Fig. S4 for a scheme of genome structure and transcript levels detection) were examined for seed formation, seed yield, germination, and growth. In these studies, we found, in contrast to the results in tomato, that the lack of Atα-DOX2 function did not provoke any visible phenotypic alteration or premature senescence. This finding could indicate that *Atα-DOX2* might differ from *Slα-DOX2* with regard to its expression characteristics. Alternatively, these results may reflect species-specific differences distinguishing tomato and Arabidopsis. In this respect, a functional redundancy of Atα-DOX2 could be implied. A candidate to substitute for the *Atα-DOX2* defect was *Atα-DOX1*, which is the closest gene homolog to *Atα-DOX2* in Arabidopsis. This possibility was tested by generating a double Arabidopsis mutant lacking both enzymatic activities: α-DOX1 and α-DOX2 (see Supplemental Fig. S4 for the genome structure and transcript levels detection). As found in the single α-DOX mutants, the examined double mutants did not show any visible phenotypic alteration compared to wild-type plants (data not shown), which demonstrated that the lack of phenotype in the *α-dox2* mutants was not due to a compensatory effect of Atα-DOX1.

Atα-DOX2 Is Expressed in Different Plant Tissues during Plant Development

In order to compare the expression patterns of *Atα-DOX2* and *Slα-DOX2*, the expression of *Atα-DOX2* was analyzed using plants expressing an *Atα-DOX2* promoter GUS (*Atα-DOX2*::*GUS*) construct and by northern analysis. *Atα-DOX2* was expressed in cotyledons, young leaves, and hypocotyls of transgenic seedlings (Fig. 5A). Expression decreased as plants matured and was only occasionally detected in leaves of flowering plants (Fig. 5A). In contrast to leaves, no significant levels of GUS activity were observed in roots at any developmental stage. Detailed examina-

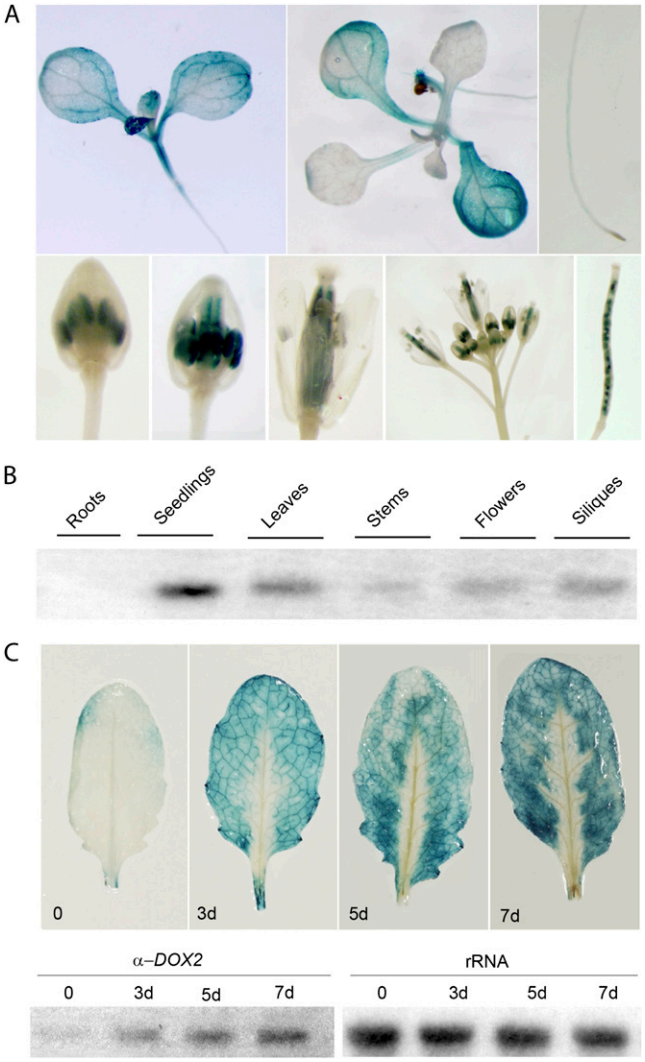


Figure 5. Expression of *Atα-DOX2*. A, Histochemical localization of GUS gene expression in transgenic plants containing an *Atα-DOX2*::*GUS* chimeric construct. Bright-field micrographs reveal in blue the presence of GUS enzyme activity in seedlings and mature tissues of transgenic plants. B, RNA was extracted from different plant organs of healthy untreated plants. Blots were hybridized with riboprobes derived from an *Atα-DOX2* cDNA. C, *Atα-DOX2* expression during detachment of mature Arabidopsis leaves. Histochemical localization of GUS gene expression in adult leaves of *Atα-DOX2*::*GUS* transgenic plants during a 1-week period after detachment. RNA blots were hybridized with riboprobes derived from an *Atα-DOX2* cDNA. Loading control was analyzed by ethidium bromide staining followed by hybridization against an 18S rRNA radioactive probe.

tion by GUS staining of flowers and siliques revealed that *Atα-DOX2* was expressed in anthers and ovules prior to fertilization as well as in developing seeds (Fig. 5A). The GUS activity found in the examined transgenic lines correlated with the accumulation of *Atα-DOX2* transcripts detected by northern blot in RNA samples prepared from different tissues of wild-type plants. Thus, examination of untreated wild-type plants revealed *Atα-DOX2* expression in seedlings, young leaves, stems, flowers, and siliques (Fig. 5B). Further examination of gene expression after stress treatments revealed that, as found for *Slα-DOX2*, the expression of *Atα-DOX2* was increased after leaf detachment. *Atα-DOX2* expression was observed 3 d after detachment and maintained up to at least 1 week (Fig. 5C). In summary, the results from these studies revealed significant similarities in the expression pattern of *Atα-DOX2* and *Slα-DOX2*.

Complementation of the Tomato *div* Mutant by Arabidopsis or Tomato *α-DOX2*

To obtain further insight into the functionality of the α-DOX2 α-dioxygenases, we tested whether Arabidopsis Atα-DOX2 could substitute for the function of the tomato enzyme in vivo. This was assessed by complementation studies of the tomato *div* mutant with the wild-type Arabidopsis *Atα-DOX2* cDNA. Transformation with a *35S*::*Slα-DOX2* construct was performed and used as a control in these experiments. Two transgenic lines with constitutive expression of *Atα-DOX2* or *Slα-DOX2* were selected (see Supplemental Fig. S5 for characterization of transgene expression). The phenotype of a transgenic line for each construct is shown in Figure 6. The phenotypic alterations that characterize *div* seedlings (delayed development and anthocyanin accumulation in seedlings; Fig. 3C) were reversed by stable transformation of tomato *div* mutant plants with a *35S*::*Atα-DOX2* construct. Complementation was also obtained by transformation with a *35S*::*Slα-DOX2* construct (Fig. 6, A and B). However, in mature plants, clear differences between transgenic and wild-type plants were detected in traits such as the morphology of the leaves, the distance between internodes, the number of lateral shoots, and the number of fruit locules. Thus, phenotypic alterations of *div* were only partially complemented in adult plants (Fig. 6, C–H), possibly due to a dose-dependent effect of α-DOX2-complementation. Independently of this, these results proved that *Atα-DOX2* can substitute for the function of the *α-DOX2* tomato gene in vivo, demonstrating the functional similarity of the two enzymes and indicating that the different phenotypic effect of mutation of Slα-DOX2 and Atα-DOX2 is not due to intrinsic differences between these proteins.

DISCUSSION

α-DOX2 from Tomato and Arabidopsis Are Authentic Fatty Acid Oxygenases with Broad Substrate Specificity

Fatty acid oxygenases initiate the synthesis of a family of lipid mediators playing critical roles in physiological and pathological processes in plants and vertebrates. Up to now, two types of fatty acid oxygenases, lipoxygenases (9- and 13-lipoxygenases) and α-dioxygenases, have been identified in plants

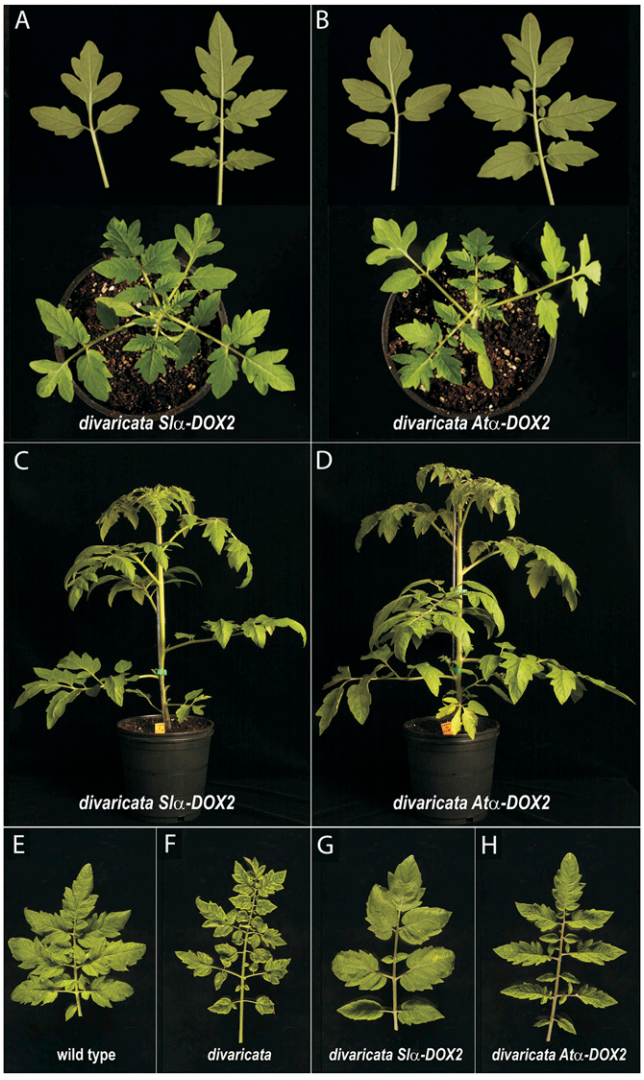


Figure 6. Complementation of tomato *div* mutant plants with *Slα-DOX2* or *Atα-DOX2* rescues the phenotypic defects of *div* plants. A, Young leaves and top view of complemented *div 35S::Slα-DOX2* plants. B, Young leaves and top view of complemented *div 35S::Atα-DOX2* plants. C, Lateral view of adult complemented *div 35S::Slα-DOX2* plants (5 weeks old). D, Lateral view of adult complemented *div 35S::Atα-DOX2* plants (5 weeks old). E, Adult leaf from the second node below the first inflorescence of a tomato wild-type plant (5 weeks old). F, Adult leaf from the second node below the first inflorescence of tomato *div* plant (5 weeks old). G, Adult leaf from the second node below the first inflorescence of tomato *div 35S::Slα-DOX2* plant (5 weeks old). H, Adult leaf from the second node below the first inflorescence of a tomato *div 35S::Atα-DOX2* plant (5 weeks old). See Figure 3 for phenotype of wild-type tomato and *div* seedlings and 5-week-old adult plants.

(Hamberg et al., 2005; Liavonchanka and Feussner, 2006). Previous characterization of the α-DOX1 α-dioxygenases from tobacco and Arabidopsis revealed their activity catalyzing the incorporation of molecular oxygen at the α-methylene carbon atom of fatty acids to generate 2*R*-hydroperoxy fatty acids derivatives (Hamberg et al., 1999). In this study, we focused our interest on the characterization of a second group of predicted α-dioxygenases, α-DOX2, that cluster as a phylogenetic group distinct from the first identified α-DOX1 (Supplemental Fig. S1). Tomato (Slα-DOX2) and Arabidopsis (Atα-DOX2) α-DOX2 were selected here to examine their catalytic activity and determine the nature of their enzymatic products. Biochemical characterization of recombinant proteins from heterologously expressed *Slα-DOX2* and *Atα-DOX2* genes, demonstrated the α-dioxygenase activity of Slα-DOX2 and Atα-DOX2. The proteins characterized catalyzed the oxygenation of fatty acids to form the same products as α-DOX1, namely, the 2*R*-hydroxy fatty acid and the corresponding one-carbon atom chain shortened aldehyde (Hamberg et al., 1999). The substrate specificity of the two examined recombinant α-DOX2 proteins appeared to be broad, as all tested fatty acids possessing chain lengths from C14 to C30, which included fully saturated fatty acids to fatty acids possessing up to three carbon double bonds, were all substrates for oxygenation. In contrast, the characterized type-1 α-dioxygenases from rice and Arabidopsis have been shown to possess a much more pronounced substrate preference for α-linolenic acid, linoleic acid, and oleic acid (Liu et al., 2006; Koszelak-Rosenblum et al., 2008). A number of conserved amino acid residues that are essential for catalytic activity in type-1 α-dioxygenases and cyclooxygenases, namely, the distal and proximal His residues involved in binding of heme, and the hydrogen-abstracting Tyr (His-163, His-389, and Tyr-389 in Atα-DOX1; Sanz et al., 1998; Liu et al., 2004) are conserved in type-2 α-dioxygenases as well (His-157, His-381, and Tyr-378 in AtDOX2 and His-157, Tyr-379, and His-382 in Slα-DOX2; Hamberg et al., 2002). In summary, our results indicate that the two type-2 α-dioxygenases characterized in this study are authentic α-dioxygenases that likely function in an enzymatically similar manner as type-1 α-dioxygenases.

Tomato and Arabidopsis α-*DOX2* Genes Are Expressed during Development and Share a Common Expression Pattern

In addition to the catalytic activity of Slα-DOX2 and Atα-DOX2, studies on the expression of *Slα-DOX2* and *Atα-DOX2* revealed significant similarities. Thus, both genes were expressed during early development, preferentially in the aerial part of the seedlings, with expression waning as the leaf matured. Whereas a typical stimulus activating the transcription of tobacco and Arabidopsis *α-DOX1* genes, such as infection of leaves with strains of *P. syringae* (Sanz et al., 1998; Ponce de León et al., 2002) did not stimulate expression of the *Slα-DOX2* or *Atα-DOX2* genes, marked expression of both genes was induced by leaf detachment. These results revealed important similarities between *Slα-DOX2* and *Atα-DOX2*. On the other hand, the expression of *Slα-DOX2* and *Atα-DOX2* differs significantly from that previously characterized *Atα-DOX1*

(Sanz et al., 1998; Ponce de León et al., 2002) and shown here for *Slα-DOX1*, pointing out to a different role of the two types of α-dioxygenase isoforms.

Further characterization of *Atα-DOX2* expression in wild-type and *Atα-DOX2::GUS* transgenic plants revealed GUS activity in stems, stamens, ovules, and siliques. Whereas the expression of *Slα-DOX2* in these organs has not been directly addressed, the fact that the anatomy of mature plants and fruits in the *div* mutant differed from that of wild-type tomato plants suggested that as found for *Atα-DOX2*, the *Slα-DOX2* gene might also be expressed in specific cells of stems and floral organs in which the activity of the encoded Slα-DOX2 protein is required for normal development. Taken together, these findings indicated that the two characterized α-DOX2 α-dioxygenases share a similar expression pattern consistent with a common function in plant development. Moreover, the characterized pattern of expression suggests that the role of α-DOX2 is not restricted to a particular phase of development but rather exerts a specific function in different plant organs throughout plant development.

Mutation of Slα-DOX2 May Alter Plant Development by Causing Alterations in Lipid Homeostasis

That Slα-DOX2 plays a role in plant development is concluded from the phenotypic abnormalities found in *div* mutants. Further support for the role of Slα-DOX2 in plant development derived from the results showing that the *div* phenotype could be reversed by transformation with a wild-type version of the *Slα-DOX2* gene. The phenotypic differences distinguishing wild-type tomato plants from *div* mutants encompass young plants, morphology of vegetative organs in adult and senescent plants, and development of fruits, indicating that wild-type levels of Slα-DOX2 activity are required for normal growth throughout the plant life cycle.

As concluded from the characterized transcriptional changes, the defect in Slα-DOX2 activity of the *div* mutation might alter the lipid composition of the plant. Thus, a high percentage of genes changing their expression are related to lipid release, transport, and metabolism. Among these, the α-DOX2 defect modifies the expression of genes that encode enzymes mediating the synthesis and posterior modification of VLCFAs, such as *KCS6* (encoding a ketoacyl-CoA synthase catalyzing the first rate limiting step in the synthesis of VLCFAs) and *CER1* (encoding an aldehyde decarbonylase; Aarts et al., 1995; Lai et al., 2007). Attempts to pinpoint lipid biosynthetic defect(s) in *div* plants by profiling cutin monomers and surface lipids have been initiated but so far produced no unequivocal result. For example, no obvious differences in the content of VLCFAs or 2-hydroxy fatty acids in cutin from leaves of wild-type or *div* plants were observed. In addition to its lipidic constituents, cutin and suberin contains low amounts of phenylpropanoids (Molina et al., 2006; Mintz-Oron et al., 2008), which might have altered content as concluded from the strong accumulation of anthocyanins (a class of phenylpropanoids) in *div* plants. Further biochemical examination will be required to examine these possibilities and to determine the nature of the lipidic changes of the *div* plants. Based on the overall alteration of the plant morphology, it is likely that changes in hormonal levels or transcription factors regulating development may also contribute to the *div* phenotype. Potential candidates to be examined in this respect are several transcription factors found to be induced in our microarray results when examined with a more relaxed stringency ($0.1 > FDR > 0.05$) and a gene encoding a noncoding RNA that is downregulated in *div* plants (Supplemental Table S1).

Significant Differences Regarding the Effect of Inactivating α-DOX2 Function Distinguish Tomato and Arabidopsis Plants

That *Atα-DOX2* knockout plants did not display visible defects in growth and development stands in clear contrast to the phenotype observed in tomato plants. However, results showing that constitutive expression of *Atα-DOX2* reverses the phenotypic alterations of the *div* mutants demonstrated the functional similarity of Slα-DOX2 and Atα-DOX2. The different phenotypes associated with the absence of α-DOX2 in tomato and Arabidopsis suggest that additional differences related to the function of α-DOX2 distinguish these two plant species. As a possibility, the α-DOX2 defect could be more easily substituted in Arabidopsis than in tomato plants by other enzymatic systems. A candidate for this could be the α-DOX1 protein. However, results shown here revealed that this is not the case as a double *α-dox1 α-dox2* Arabidopsis mutant does not show any visible phenotypic modification or senescence alteration. Alternatively, the differences between tomato and Arabidopsis could be due to intrinsic changes in the composition of the lipid structures influenced by the α-DOX2 function or in the importance of such lipidic components in the development of these two types of plants. As reported (Franke et al., 2005; Nawrath, 2006; Mintz-Oron et al., 2008), the amount and the composition of cutin and likely of other lipid structures that may be affected in the *div* mutant could differ between tomato and Arabidopsis plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum*) wild-type cultivar Condine Red and *α-DOX2 div* mutant were provided by the Tomato Genetics Resource Center (TGRC; University of California, Davis). Sterilized tomato seeds were grown in vertically oriented square petri dishes, containing 0.5× Murashige and Skoog (MS), pH 6.0, 2% Suc (w/v), and 1.5% w/v agar (Bacto Agar; Becton-Dickinson). Growth conditions were 16 h of light, 8 h of dark, and 22°C. Seedlings were transplanted to individual pots and grown in the greenhouse. Arabidopsis (*Arabidopsis thaliana*) wild-type and transgenic *Atα-DOX2::GUS* plants used in this study were derived from Arabidopsis plant ecotype Columbia (Col-0). Insertion mutants used were identified using the SIGnAL T-DNA Express Arabidopsis gene mapping tool (http://signal.salk.edu/).

SALK lines SALK_005633 (*α-dox1-1*), SALK_029547 (*α-dox2-1*), and SALK_089649 (*α-dox2-2*) were distributed by the Nottingham Arabidopsis Stock Centre (http://Arabidopsis.info). Homozygous insertion mutants were identified by PCR using T-DNA and gene-specific primer sets as described on the T-DNA Express homepage. Sterilized Arabidopsis seeds were vernalized for 3 d at 4°C and grown at 16 h of light, 8 h of dark, and 22°C in petri dishes containing 0.5× MS, pH 6.0, 1.5% Suc (w/v), and 0.8% (w/v) agar (Bacto Agar). For assays with mature plants, seeds were sown on soil and vernalized for 3 d at 4°C and then grown in chamber (22°C, 70% relative humidity, 250 μE m^{-2} s^{-1} fluorescent lighting) under a 14 h light/10 h dark photoperiod.

Sequence Alignment and Phylogenetic Relationship Analysis of Plant α-Dioxygenases

Twenty-one plant α-dioxygenases were aligned using the ClustalW2 program (Larkin et al., 2007) and subjected to phylogenetic analysis by the neighbor-joining and maximum parsimony methods using the PHYLIP package (Felsenstein, 1989) through the facilities of the Mobyle platform from the Institut Pasteur server (http://mobyle.pasteur.fr/cgi-bin/portal.py). To maximize the statistical significance of the phylogenetic trees generated by the distance and parsimony methods, 1,000 bootstrap replicates were obtained by both methods.

Cloning and Expression of Tomato and Arabidopsis α-DOX2

Recombinant baculoviruses expressing tomato (Slα-DOX2) and Arabidopsis (Atα-DOX2) α-DOX2 were generated using the Bac-to-Bac baculovirus expression system (Invitrogen). *Slα-DOX2* cDNA was isolated from a tomato cDNA library. Clone R16142 containing full-length *Atα-DOX2* cDNA was obtained from the Arabidopsis Biological Resource Center (Ohio State University). Slα-DOX2 and Atα-DOX2-encoding cDNAs (GenBank accession nos. AJ850958 and AY081283, respectively) were excised from their host plasmids and ligated into the pFastBac vector. Correct cloning of the insert was verified by sequence analysis. The recombinant plasmids were transferred into DH10Bac *Escherichia coli* cells containing the baculovirus shuttle vector bMON14272 and the helper plasmid pMON7124. Recombinant bacmid DNAs were prepared from positive bacterial clones, and recombinant baculovirus was obtained by transfection of the bacmids into High Five insect cells according to the manufacturer's instructions. In contrast to the enzymatic characterization of Atα-DOX1 (Sanz et al., 1998) the heterologous expression of α-DOX2 from Arabidopsis and tomato proved to be intricate. Baculovirus-mediated expression in insect cells led to a protein lacking enzymatic activity and was to a large extent present as aggregated protein. Satisfactory enzyme expression with enzyme activity was obtained when the cells were supplemented with hemin, added concomitantly to infection of the cells with baculovirus.

Slα-DOX2 and Atα-DOX2 were expressed by infecting High Five insect cell cultures, grown at 28°C in Tc-100 medium supplemented with 10% fetal calf serum, 10 μM hemin, and the recombinant baculovirus. At 48 h after infection, cells were collected by centrifugation (5 min, 3,000*g*), washed twice with Dulbecco's phosphate-buffered saline (pH 7.4), divided in aliquots, and pelleted by centrifugation (5 min, 3,000*g*). Cell pellets were snap-frozen in liquid nitrogen and stored at −80°C. In order to limit enzymatic inactivation, broken cell preparations from recently thawed frozen cell pellets were used to determine enzyme activity in this study. Total protein lysates of α-dioxygenase-expressing High Five insect cells were prepared in sample buffer (30 mM Tris, pH 6.8, 0.5% SDS, 0.5% β-mercaptoethanol, 5% glycerol, 1 mM EDTA, 1× protease inhibitor cocktail [Sigma-Aldrich P-2714], and 0.005% bromphenol blue), and separated by SDS-PAGE (9% cross-linked gels) at 100 V for 3 h in a Bio-Rad gel electrophoresis system (Lowry et al., 1951). Proteins were stained with Coomassie Brilliant Blue. The apparent M_r of the recombinant proteins was determined using M_r marker proteins (Precision Plus protein standards; Bio-Rad). Total protein content was determined by the method of Bradford using cell homogenates prepared in 0.1 M Tris buffer, pH 7.4, with 0.1% Triton X-100 (Bradford, 1976).

α-Dioxygenase Activity

α-Dioxygenase activity was measured by using a Clark-type oxygen electrode (Hansatech Instruments). High Five insect cell pellets containing Slα-DOX2 or Atα-DOX2 (approximately 100 μg total protein) were thawed in 50 μL 0.1 M Tris, pH 7.4, passed five times through a 100-μL Hamilton syringe, and rapidly brought to room temperature. The broken cell preparations were added to the measuring cell containing 1.5 mL 0.1 M Tris, pH 7.4, 100 μM fatty acid substrate, and 100 μM *tert*-butyl-hydroperoxide. Oxygen consumption was recorded at room temperature, and the rate of enzyme activity calculated as nmol oxygen consumed during the first minute per mg protein. The oxygenase activities were determined using saturated fatty acids ranging in chain length from 14 to 30 carbons and the following unsaturated fatty acids: 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic (C16:3), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and 11(*Z*)-eicosenoic acid (C20:1). Fatty acids with carbon chain lengths ranging from 14 to 18 were added from ethanol stocks (final assay concentration of ethanol 0.05%), whereas stock solutions of the less soluble C20 to C30 fatty acids were prepared in 1% Tween 20 containing 0.1 N NaOH (final assay concentration of Tween 20 was 0.01%).

Enzyme Incubations and Product Identification

For identification of reaction products, homogenates of High Five cells (approximately 6×10^6 cells) containing Atα-DOX2 or Slα-DOX2 were incubated with palmitic, stearic, linolenic, or arachidic acids (100 μM) in 8 mL of 0.1 M potassium phosphate buffer, pH 7.0, at 23°C under oxygen atmosphere. After 30 min, 20 mL of 30 mM methoxyamine hydrochloride solution in methanol was added to derivatize aldehydes. After 1 h at 23°C, the products were extracted with diethyl ether, derivatized with diazomethane and trimethylchlorosilane, and analyzed by GC-MS. GC-MS analysis was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph equipped with a phenylmethylsiloxane capillary column (12 m, film thickness 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas.

Steric analysis of 2-hydroperoxide derivatives generated following incubation of palmitic and linolenic acids with Atα-DOX2 or Slα-DOX2 was carried out following reduction with sodium borohydride, methylesterification, and isolation of the 2-hydroxyesters by thin-layer chromatography. (−)-Menthoxycarbonyl derivatives were prepared by treatment of the 2-hydroxyesters with (−)-menthylchloroformate/toluene/pyridine (10:10:3, v/v/v) at 23°C for 15 h and purified by thin-layer chromatography. Separation of the (−)-menthoxycarbonyl derivatives of 2(*R*)- and 2(*S*)-hydroxyesters was achieved by GC-MS under the conditions described above. The corresponding derivatives of 2(*R*)- and 2(*R*,*S*)-hydroxypalmitic acids and of 2(*R*)- and 2(*R*,*S*)-hydroxylinolenic acids (Lipidox) were used as references.

Plant Treatments and RNA Isolation

For microarray analyses, hypocotyls and cotyledons of 7-d-old tomato seedlings grown on MS medium were excised and used to compare gene expression between the wild-type control and the *div* mutant. For detachment assays, mature leaves were detached at the petiole from the stem using a forceps and placed onto a Whatman paper filter in water in a 14-cm-diameter petri dish. The dish was placed in a growth chamber in normal lighting conditions, and leaves were collected at days 0, 1, 3, 5, and 7 after detachment. In all cases, collected tissues were frozen in liquid nitrogen and stored at −80°C until analysis. Tomato total RNA was isolated by using the RNeasy plant mini kit (Qiagen), whereas Arabidopsis total RNA was isolated according to Logemann et al. (1987).

Microarray Hybridization and Analysis

Gene expression of wild-type tomato seedling aerial parts grown on MS medium versus *α-DOX2 div* mutant was compared using the Affymetrix GeneChip Tomato Genome Array. This array consists of over 10,000 tomato probe sets to interrogate over 9,200 tomato transcripts. More information can be found at the Affymetrix homepage (http://www.affymetrix.com/products_services/arrays/specific/tomato.affx). Total RNA was isolated from three independent biological replicates. RNA samples from the wild type and *div* mutant were quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technology) and assessed using an Agilent 2100 bioanalyzer (Agilent Technologies). cDNA was synthesized from 4 μg of total RNA using one-cycle target labeling and control reagents (Affymetrix) to produce biotin-labeled cRNA. The cRNA preparation (15 μg) was fragmented at 94°C for 35 min into 35 to 200 bases in length. If the quality control was correct, 5 μg of fragmented cRNA were hybridized to the Tomato Genome Array. Each sample was added to a hybridization solution containing 100 mM MES, 1 M Na^+, and 200 mM of EDTA in the presence of 0.01% of Tween 20 to a final cRNA

concentration of 0.05 μg/mL. Hybridization was performed for 16 h at 45°C. Each microarray was washed and stained with streptavidin-phycoerythrin in a Fluidics station 450 (Affymetrix) and scanned at 1.56-μm resolution in a GeneChipScanner 3000 7G System (Affymetrix). Data analyses were performed using GeneChip Operating Software. Arrays were hybridized, stained, washed, and screened for quality at the Genomics Service of the Centro Nacional de Biotecnología (Consejo Superior de Investigaciones Científicas). The robust multiarray analysis algorithm was used for background correction, normalization, and expression level summarization (Irizarry et al., 2003). Raw data and normalized data were deposited at ArrayExpress data library (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-2265. Differentially expressed transcripts were determined using the rank products method (Breitling et al., 2004). The multiple testing problem inherent to microarray experiments was corrected using the FDR method (Benjamin and Hochberg, 1995; Reiner et al., 2003). An FDR of 5% means that only 5% or less of the transcripts up to this position is expected to be observed by chance (false positives), with the remaining 95% being transcripts that are indeed significantly affected (true positives). Significantly up-regulated and down-regulated transcripts obtained in seedlings aerial parts of *div* mutant compared with the wild type (at FDR of 5%), represented in red and green, respectively, are listed in Supplemental Table S1 online in ascending order of FDR. Additionally, fold change representing differential expression ratio is listed for each probe. In order to improve the annotation of transcripts (at FDR of 21%), the most closely related Arabidopsis homologous loci found using the BLAST algorithm (Zhang et al., 2000) against the National Center for Biotechnology Information nonredundant database of 2009-07-09 (lower E-value) are listed for each probe. Statistical analysis and graphical visualization of data were performed with the interactive tool FIESTA (http://bioinfogp.cnb.csic.es/tools/FIESTA).

Analysis of Gene Expression

RT-PCR analyses were performed with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using the Titan One Tube RT-PCR system (Roche Applied Science) as specified by the manufacturer. Total RNA was treated with DNase TURBO DNA-free (Ambion) to remove contaminating DNA. A quantity (100 ng) of this RNA was used in each one-step RT-PCR procedure. Primers used and the lengths of tomato and Arabidopsis amplification products are described in Supplemental Tables S2 and S3, respectively, online. Tomato gene GAPDH encoding glyceraldehyde 3-phosphate dehydrogenase and Arabidopsis cytoplasmic ribosomal protein L3A were used as internal standards, respectively. For northern blots, RNA (5 μg per lane) was analyzed in agarose-formaldehyde gels, transferred to Hybond N membranes, and hybridized to single-stranded riboprobes following standard procedures (Sambrook et al., 1989). Radioactive probes were prepared for Arabidopsis *α-DOX2* from clone R16142 containing full-length cDNA. The amount of loaded RNA was verified by addition of ethidium bromide to the samples and photography under UV light after electrophoresis, followed by hybridization to 18S rRNA (Ruiz-García et al., 1997). Blots shown are representative examples of the results obtained in three independent experiments.

Construction of Transgenic Lines and Analyses of GUS Activity

Genomic sequence extending to approximately 1 kb from the translational start site of the Arabidopsis *Atα-DOX2* gene was PCR amplified from wild-type Col-0 using Expand High Fidelity polymerase (Roche). The forward and reverse primers used were 5′-GCTAATAATTCCGAGGGACAGAA-3′ and 5′-CTGTTTTACATATCATTTCTTTTACGG-3′, respectively. The resulting PCR fragment was inserted into the plasmid pGEM-T Easy vector system I (Promega) and sequenced to ensure correct amplification. The promoter sequence was fused to the coding region of the GUS gene present in the plasmid pBI101.2, which confers resistance to kanamycin in planta, introduced into *Agrobacterium tumefaciens*, and transferred into Col-0 wild-type plants. Homozygous transgenic lines were selected for these studies, and examination of GUS activity was performed as described (Malamy and Benfey, 1997).

Generation of Tomato Transgenic Lines

Slα-DOX2 and *Atα-DOX2* genes were excised from their host plasmids and cloned into pGSJ780A and pROK binary vectors harboring a cauliflower mosaic virus 35S promoter resulting in *35S::Slα-DOX2* and *35S::Atα-DOX2* constructs, respectively. These constructs were introduced into *A. tumefaciens* and transferred into tomato *div* mutant plants according to McCormick (1991). Two different homozygous transgenic lines were selected in each case. Genotype of transgenic plants was verified by PCR amplification and sequencing. Transgene overexpression was confirmed by RT-PCR (Supplemental Fig. S5). Amplification of *Atα-DOX2* was performed with oligonucleotides 5′-ACACCAATCTTGTGGCGCATT-3′ and 5′-CTTCATCATCTGTCAACTCTTCC-3′ generating a 221-bp amplicon.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ850958 (tomato *α-DOX2* cDNA) and FN428743 (tomato *α-DOX2* genomic sequence).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic analysis of available α-dioxygenases.

Supplemental Figure S2. Heterologous expression of Slα-DOX2 and Atα-DOX2 in insect cells.

Supplemental Figure S3. Schematic representation of the α-dioxygenation reaction catalyzed by Slα-DOX2 and Atα-DOX2.

Supplemental Figure S4. Scheme of *Atα-DOX1* and *Atα-DOX2* genomic structures and T-DNA insertion mutants.

Supplemental Figure S5. *α-DOX2* expression in tomato wild-type, *div* mutant, and *div* transgenic lines.

Supplemental Table S1. Differentially expressed genes in seedlings of tomato *div* mutant versus wild type.

Supplemental Table S2. Sets of primers used to examine tomato gene expression.

Supplemental Table S3. Sets of primers used to examine Arabidopsis gene expression.

ACKNOWLEDGMENTS

We thank Michael Bartsch, Pilar Cubas, and Javier Paz-Arés for critical reading of the manuscript; M. Irigoyen for help with phenotypic analyses; R. Piqueras and M. Peinado for help with in vitro plant growth; I. Poveda for expert photography; and Gunvor Hamberg and Tomas Cascón for excellent technical assistance. We also thank G. Martin for the tomato cDNA library. The tomato wild-type cultivar Condine Red and *div* mutant were provided by the Tomato Genetics Resource Center. The Arabidopsis *α-DOX2* cDNA was from the Arabidopsis Biological Resource Center (Ohio State University). The T-DNA insertion lines used in these studies were from the SALK collection and were obtained from the Nottingham Arabidopsis Stock Centre. Microarray analyses were performed at the genomic facilities of the Centro Nacional de Biotecnología (http://www.cnb.csic.es/~genomica).

Received July 21, 2009; accepted September 14, 2009; published September 16, 2009.

LITERATURE CITED

Aarts MGM, Keijzer CJ, Stiekema WJ, Pereira A (1995) Molecular characterization of the CER1 gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell **7:** 2115–2127

Benjamin Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc, B **57:** 289–300

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Breitling R, Armengaud P, Amtmann A, Herzyk P (2004) Rank products: a

simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett **573:** 83–92

Browse J (2005) Jasmonate: an oxylipin signal with many roles in plants. Vitam Horm **72:** 431–456

Farmer EE, Almeras E, Krishnamurthy V (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol **6:** 372–378

Felsenstein J (1989) PHYLIP-Phylogeny inference package (version 3.2). Cladistics **5:** 164–166

Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol **53:** 275–297

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L (2005) Apoplastic polyesters in *Arabidopsis* surface tissues. A typical suberin and a particular cutin. Phytochemistry **66:** 2643–2658

Gepstein S, Sabehi G, Carp MJ, Hajouj T, Nesher MF, Yariv I, Dor C, Bassani M (2003) Large-scale identification of leaf senescence-associated genes. Plant J **36:** 629–642

Guo Y, Gan S (2005) Leaf senescence: signals, execution, and regulation. Curr Top Dev Biol **71:** 83–112

Hamberg M, Ponce de León I, Rodríguez MJ, Castresana C (2005) α-Dioxygenases. Biochem Biophys Res Commun **338:** 169–174

Hamberg M, Ponce de León I, Sanz A, Castresana C (2002) Fatty acid α-dioxygenases. Prostaglandins Other Lipid Mediat **68-69:** 363–374

Hamberg M, Sanz A, Castresana C (1999) α-Oxidation of fatty acids in higher plants. Identification of a pathogen-inducible oxygenase (piox) as an α-dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. J Biol Chem **274:** 24503–24513

Hamberg M, Sanz A, Rodríguez MJ, Calvo AP, Castresana C (2003) Activation of the fatty acid α-dioxygenase pathway during bacterial infection of tobacco leaves. Formation of oxylipins protecting against cell death. J Biol Chem **278:** 51796–51805

Hermsmeier D, Schittko U, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiol **125:** 683–700

Howe GA, Schilmiller AL (2002) Oxylipin metabolism in response to stress. Curr Opin Plant Biol **5:** 230–236

Irizarry RA, Hobbs B, Colllin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:** 249–264

Kachroo A, Kachroo P (2009) Fatty acid-derived signals in plant defense. Annu Rev Phytopathol **47:** 153–176

Kandel S, Sauveplane V, Olry A, Diss L, Benviste I, Pinot F (2006) Cytochrome P450-dependent fatty acid hydroxylases in plants. Phytochem Rev **5:** 359–372

Koeduka T, Matsui K, Hasegawa M, Akakabe Y, Kajiwara T (2005) Rice fatty acid α-dioxygenase is induced by pathogen attack and heavy metal stress: activation through jasmonate signaling. J Plant Physiol **162:** 912–920

Koszelak-Rosenblum M, Krol AC, Simmons DM, Goulah CC, Wroblewski L, Malkowski MG (2008) His-311 and Arg-559 are key residues involved in fatty acid oxygenation in pathogen-inducible oxygenase. J Biol Chem **283:** 24962–24971

Lai C, Kunst L, Jetter R (2007) Composition of alkyl esters in the cuticular wax on inflorescence stems of *Arabidopsis thaliana cer* mutants. Plant J **50:** 189–196

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, López R, et al (2007) ClustalW and ClustalX version 2.0. Bioinformatics **23:** 2947–2948

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol **57:** 405–430

Liavonchanka A, Feussner I (2006) Lipoxygenases: occurrence, functions and catalysis. J Plant Physiol **163:** 348–357

Liu W, Rogge CE, Bambai B, Palmer G, Tsai AL, Kulmacz RJ (2004) Characterization of the heme environment in *Arabidopsis thaliana* fatty acid α-dioxygenase-1. J Biol Chem **279:** 29805–29815

Liu W, Wang LH, Fabian P, Hayashi Y, McGinley CM, van der Donk WA, Kulmacz RJ (2006) *Arabidopsis thaliana* fatty acid alpha-dioxygenase-1: evaluation of substrates, inhibitors and amino-terminal function. Plant Physiol Biochem **44:** 284–293

Logemann J, Schell J, Willlmitzer L (1987) Improved method for isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Lowry OH, Roseborough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem **193:** 265–273

Malamy J, Benfey PN (1997) Analysis of SCARECROW expression using a rapid system for assessing transgene expression in Arabidopsis roots. Plant J **12:** 957–963

McCormick S (1991) Transformation of Tomato with *Agrobacterium tumefaciens*, Vol B. Kluwer, Amsterdam

Mintz-Oron S, Mandel T, Rogachev I, Feldberg L, Lotan O, Yativ M, Wang ZY, Jetter R, Venger I, Adato A, et al (2008) Gene expression and metabolism in tomato fruit surface tissues. Plant Physiol **147:** 823–851

Molina I, Bonaventure G, Ohlrogge J, Pollard M (2006) The lipid polyester composition of *Arabidopsis thaliana* and *Brassica napus* seeds. Phytochemistry **67:** 2597–2610

Nawrath C (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol **9:** 281–287

Ponce de León I, Sanz A, Hamberg M, Castresana C (2002) Involvement of the Arabidopsis α-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. Plant J **29:** 61–62

Reiner A, Yekutieli D, Benjamin Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics **19:** 368–375

Ruiz-García L, Madueño F, Wilkinson M, Haughn G, Salinas J, Martínez-Zapater JM (1997) Different roles of flowering-time genes in the activation of floral initiation genes in *Arabidopsis*. Plant Cell **9:** 1921–1934

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanz A, Moreno JI, Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. Plant Cell **10:** 1523–1537

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, et al (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31:** 279–292

Shibata D, Axelrod B (1995) Plant lipoxygenases. J Lipid Mediat Cell Signal **12:** 213–228

Stevens MA, Rick CM (1986) Genetics and breeding. *In* JG Atherton, J Rudich, eds, The Tomato Crop, A Scientific Basis for Crop Improvement. Chapman and Hall, New York, pp 35–100

van der Biezen EA, Brandwagt BF, van Leeuwen W, John J, Nijkamp J, Hille J (1996) Identification and isolation of the FEEBLY gene from tomato by transposon tagging. Mol Genet Genomics **251:** 267–280

Weber H (2002) Fatty acid-derived signals in plants. Trends Plant Sci **7:** 217–224

Wei J, Tirajoh A, Effendy J, Plant AL (2000) Characterization of salt-induced changes in gene expression in tomato (*Lycopersicon esculentum*) roots and the role played by abscisic acid. Plant Sci **159:** 135–148

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol **7:** 203–214

the plant journal

SEB Society for Experimental Biology

The Plant Journal (2011) **67**, 447–458 doi: 10.1111/j.1365-313X.2011.04608.x

Antagonistic role of 9-lipoxygenase-derived oxylipins and ethylene in the control of oxidative stress, lipid peroxidation and plant defence

Miguel A. López[1,†], Jorge Vicente[1,†], Satish Kulasekaran[1], Tamara Vellosillo[1], Marta Martínez[1], Maria L. Irigoyen[1], Tomas Cascón[1], Gerard Bannenberg[1], Mats Hamberg[2] and Carmen Castresana[1,*]

[1]*Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, CSIC, Campus Universidad Autónoma, Cantoblanco, E-28049 Madrid, Spain, and*

[2]*Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden*

Received 10 March 2011; revised 6 April 2011; accepted 6 April 2011; published online 06 June 2011.
*For correspondence (fax +34 91 5854506; e-mail ccastresana@cnb.csic.es).
†These authors contributed equally to this work.

SUMMARY

9-lipoxygenases (9-LOXs) initiate fatty acid oxygenation in plant tissues, with formation of 9-hydroxy-10,12,15-octadecatrienoic acid (9-HOT) from linolenic acid. A *lox1 lox5* mutant, which is deficient in 9-LOX activity, and two mutants *noxy6* and *noxy22* (*non-responding to oxylipins*), which are insensitive to 9-HOT, have been used to investigate 9-HOT signalling. Map-based cloning indicated that the *noxy6* and *noxy22* mutations are located at the *CTR1* (CONSTITUTIVE ETHYLENE RESPONSE1) and *ETO1* (ETHYLENE-OVERPRODUCER1) loci, respectively. In agreement, the *noxy6* and *noxy22* mutants, renamed as *ctr1-15* and *eto1-14*, respectively, showed enhanced ethylene (ET) production. The correlation between increased ET production and reduced 9-HOT sensitivity indicated by these results was supported by experiments in which exogenously added ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) impaired the responses to 9-HOT. Moreover, a reciprocal interaction between ET and 9-HOT signalling was indicated by results showing that the effect of ACC was reduced in the presence of 9-HOT. We found that the 9-LOX and ET pathways regulate the response to the lipid peroxidation-inducer singlet oxygen. Thus, the massive transcriptional changes seen in wild-type plants in response to singlet oxygen were greatly affected in the *lox1 lox5* and *eto1-14* mutants. Furthermore, these mutants displayed enhanced susceptibility to both singlet oxygen and *Pseudomonas syringae* pv. *tomato*, in the latter case leading to increased accumulation of the lipid peroxidation product malondialdehyde. These findings demonstrate an antagonistic relationship between products of the 9-LOX and ET pathways, and suggest a role for the 9-LOX pathway in modulating oxidative stress, lipid peroxidation and plant defence.

Keywords: oxylipins, ethylene, oxidative stress, plant defence, Arabidopsis.

INTRODUCTION

Oxylipins are lipid-derived molecules that are ubiquitous in eukaryotes, and whose importance in controlling physiological and pathological processes in plants is being recognized (Savchenko *et al.*, 2010; López *et al.*, 2008). Enzymatic synthesis of oxylipins is initiated by incorporation of oxygen into a fatty acid molecule, catalysed by the activities of 9- and 13-lipoxygenases or α-dioxygenases (Andreou *et al.*, 2009; Hamberg *et al.*, 2005). The primary product is a fatty acid hydroperoxide, which is subsequently modified by an array of secondary enzymatic activities to generate an extensive family of metabolites (Mosblech *et al.*, 2009). Production of oxylipins from polyunsaturated fatty acids can also take place non-enzymatically in the presence of singlet oxygen or by free radical-mediated oxygenation (Durand *et al.*, 2009; Hamberg, 2011).

Synthesis of oxylipins is triggered at specific developmental stages, as well as by various biotic and abiotic stresses. During these responses, oxylipins were found to regulate gene expression (Stintzi *et al.*, 2001; Mueller *et al.*, 2008; Browse, 2009) and cell death (Vollenweider *et al.*, 2000; De León *et al.*, 2002; Hamberg *et al.*, 2003; Montillet *et al.*, 2005), and to exert direct anti-microbial activity (Prost

et al., 2005; Kishimoto *et al.*, 2008). Much attention has been paid to the biosynthetic pathway initiated by 13-lipoxygenases (13-LOX), and its main product, jasmonic acid (JA), which plays critical roles in fertility, resistance to necrotrophic pathogens, insect attack, wounding and establishing systemic resistance (Mandaokar and Browse, 2009; Caldelari *et al.*, 2011; Glazebrook, 2005; Onkokesung *et al.*, 2010; Koo and Howe, 2009; Truman *et al.*, 2007). A number of studies have also demonstrated the participation of oxylipins produced by the 9-LOX and α-DOX pathways in regulating plant development and defence responses (Rancé *et al.*, 1998; Vellosillo *et al.*, 2007; Gao *et al.*, 2008; Hwang and Hwang, 2010; De León *et al.*, 2002; Obregón *et al.*, 2001; Bannenberg *et al.*, 2009b). Furthermore, increasing evidence indicates a role for non-enzymatically generated oxylipins in plant defence (Loeffler *et al.*, 2005; Mueller and Berger, 2009). Nevertheless, the signalling mechanisms by which these oxylipins exert their function remain poorly understood.

Using a collection of 47 oxylipins and an *in vitro* assay, we identified three types of oxylipin-activated phenotypic alterations on root growth, i.e. root waving, loss of root apical dominance, and decreased root elongation. Among the oxylipins tested, the 9-LOX product 9(*S*)-hydroxy-10,12,15-octadecatrienoic acid (9-HOT) was the most potent inducer of root waving, and was used to identify 9-HOT-insensitive *noxy* mutants (*non-responding to oxylipins*) within a mutagenized Arabidopsis population. Studies using the 9-HOT-insensitive mutant *noxy2* and the JA-insensitive *coi1-1* mutant demonstrated that the response of plants to 9-HOT was activated by a JA-independent signalling pathway, and that the *noxy* mutants constitute a valuable tool to investigate the signalling components of the 9-HOT response (Vellosillo *et al.*, 2007).

Examination of the molecular events triggered by 9-HOT revealed accumulation of callose, production of reactive oxygen species (ROS), and transcriptional changes for genes involved in plant defence. These actions are common to the response to pathogen infection (Dangl and Jones, 2001), and are indicative of a role of 9-HOT in plant defence. In agreement with this, the 9-HOT-insensitive *noxy2* mutant showed enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*). Moreover, *noxy2* mutants showed reduced necrosis after infection compared with wild-type plants. These findings were in line with previous results showing accumulation of 9-HOT, and additional 9-LOX derivatives, during activation of a hypersensitive cell-death response protecting plants against biotrophic pathogens, as well as in the cell-death reaction caused by pathogen elicitors (Vollenweider *et al.*, 2000; Hamberg *et al.*, 2003; Göbel *et al.*, 2003; Andersson *et al.*, 2006; Rustérucci *et al.*, 1999; Montillet *et al.*, 2005).

Here, we have characterized the signalling processes regulated by the 9-LOX derivative 9-HOT. We made use of a double mutant, *lox1 lox5*, that lack 9-LOX activity, and of two 9-HOT-insensitive mutants, *noxy6* and *noxy22* (*non-responding to oxylipins*), and uncovered an antagonistic role of 9-LOX and ethylene (ET) in controlling responses involving oxidative stress, lipid peroxidation and plant defence.

RESULTS

The *noxy6* and *noxy22* mutants, which are non-responsive to 9-HOT, are constitutive ethylene mutants

The 9-HOT-insensitive mutants *noxy6* and *noxy22* were isolated by a forward genetic screen based on the root waving activity of 9-HOT. We found that both mutants failed to respond to 9-HOT by inducing root waving but did respond to oxylipins such as JA and 9-oxononanoic acid (9-Oxo-C_9), which cause a reduction of root elongation (Figure S1). In addition, *noxy6* and *noxy22* plants did not activate responses to 9-HOT such as formation of focal deposits of callose (Figure S2). Moreover, production of superoxide anion and expression of three genes up-regulated by 9-HOT, i.e. *POX* (pyridine nucleotide oxidoreductase, At5g22140), *ABC* (ABC transporter, At1g15520) and *FOX* (FAD-binding oxidoreductase, At1g26380) (Vellosillo *et al.*, 2007), were reduced in these *noxy* mutants compared with wild-type plants (Figure S2).

Genetic analysis indicated that the *noxy6* and *noxy22* mutations were monogenic and recessive. Map-based cloning showed that the *noxy6* mutation is a C → T transition at nucleotide 3544 in the *CTR1* gene, which was renamed *ctr1-15* (Figure S3). The *ctr1-15* mutation converts Leu610 to Phe in the kinase domain of the CTR1 protein, which is a negative regulator of the ET pathway (Kieber *et al.*, 1993). The *noxy22* mutation is a C → T transition that converts Pro265 to Leu in the ETO1 protein, and was renamed *eto1-14* (Figure S3). The ETO1 protein is a negative regulator of ET synthesis (Wang *et al.*, 2004). As a consequence of the *ctr1-15* and *eto1-14* mutations, the ET pathway was constitutively activated, as confirmed by the characteristic triple response of dark-grown seedlings (Figure S4) (Guzmán and Ecker, 1990). These results indicate that ET may negatively interact with 9-HOT signalling.

Ethylene impairs the responses to 9-HOT via the canonical ethylene signalling pathway

Because constitutive ET production diminishes the response to 9-HOT, we examined the effect of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) on the 9-HOT response. We found that ACC impaired root waving, and reduced the accumulation of callose and production of superoxide anion that otherwise accompanied 9-HOT treatment (Figure 1). We also tested the response of the ET-insensitive mutant *ein2-5* (Alonso *et al.*, 1999) to application of 9-HOT, either alone or in combination with ACC. We found that *ein2-5* reacted as wild-type plants do to 9-HOT by

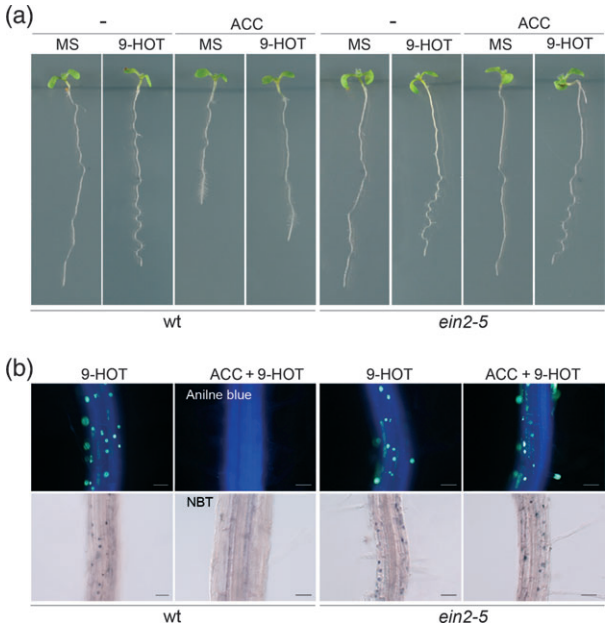


Figure 1. Characterization of wild-type and *ein2-5* plants in response to ACC and 9-HOT.
(a) Transmitted light visualization of seedlings grown for 3 days in MS medium and transferred to grow for an additional 3 days on MS medium containing 9-HOT (25 μM), ACC (2 μM) or 9-HOT in combination with ACC.
(b) Staining of roots treated as above. Fluorescence visualization of callose deposition in roots stained with aniline blue (upper panels), and transmitted light visualization of superoxide ions in roots stained with nitroblue tetrazolium (lower panels).

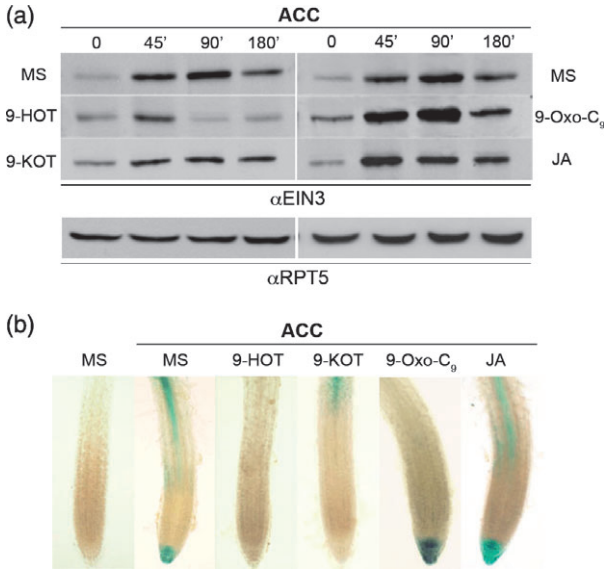


Figure 2. Analyses of ET signalling activation.
(a) Analyses of EIN3 protein accumulation in 6-day-old seedlings of *35S:EIN3-GFP* transgenic lines grown in MS medium and transferred to MS medium containing ACC (2 μM) alone or in combination with the oxylipins 9-HOT, 9-KOT, JA or 9-Oxo-C_9 (25 μM). Proteins were extracted and examined at the times indicated. Blots were hybridized to an antibody against EIN3. Anti-RPT5 was used as a loading control.
(b) Histological examination of GUS activity in transgenic lines containing the ethylene-responsive reporter *EBS:GUS*. GUS staining was performed 24 h after treatment with ACC alone or in combination with the oxylipins 9-HOT, 9-KOT, JA or 9-Oxo-C_9 (25 μM).

inducing root waving, callose deposition and ROS production. However, the effect of ACC on 9-HOT-activated root responses was impaired in the *ein2-5* mutant. These results indicated that activation of the 9-HOT responses examined does not require an active ET signalling pathway, but that the inhibitory effect of ACC on 9-HOT signalling is exerted through the canonical ET signalling pathway. Further, analysis of genes up-regulated by 9-HOT in *ein2-5* plants revealed enhanced accumulation of the three transcripts examined (*POX*, *ABC* and *FOX*) compared with wild-type plants (Figure S5). These results support a negative regulatory role of ET on the 9-HOT signalling pathway.

9-HOT interferes with activation of the ET signalling pathway

Because enhanced ET production inhibits 9-HOT signalling, it was of interest to examine whether, reciprocally, 9-HOT could antagonize ET signalling. In support of this idea, we noted that the inhibitory effect of ACC on root growth was diminished in the presence of 9-HOT (Figure 1a). To evaluate the effect of 9-HOT on the ET pathway, we made use of a well-characterized transgenic line *35S:EIN3-GFP* that shows constitutive expression of EIN3, a key transcription factor that is stabilized in response to ET to activate gene expression (Guo and Ecker, 2003). As shown in Figure 2(a), accumulation of EIN3–GFP increased strongly on application of ACC, and the increase was clearly reduced when 9-HOT was applied simultaneously with ACC. Further support for the effect of 9-HOT on the ET pathway was obtained using *EBS:*β-glucoronidase (GUS) transgenic plants, in which expression of the GUS gene is driven by a synthetic EIN3-responsive promoter (Stepanova *et al.*, 2007). As shown in Figure 2(b), no detectable expression of *EBS:GUS* was observed in roots of untreated plants, but ACC treatment resulted in a significant increase in GUS in root tips and in the elongation zone of the roots. The activation of *EBS:GUS* was reduced to a great extent when ACC was applied in combination with 9-HOT, proving that exogenous 9-HOT interferes with activation of the ET pathway.

In addition to 9-HOT, we explored the action of a second root waving oxylipin, i.e. 9-keto-10,12,15-octadecatrienoic acid (9-KOT), as well as of oxylipins that cause a reduction of root elongation, such as JA and 9-Oxo-C_9. We found that 9-KOT reduced the ACC-induced accumulation of EIN3, but to a smaller extent than 9-HOT, whereas JA and 9-Oxo-C_9 led to an increase in EIN3 compared with the level observed when ACC alone was applied (Figure 2a). Accordingly, activation of the *EBS:GUS* construct was significantly

reduced when ACC was applied in combination with 9-KOT, whereas no decrease of GUS activity was detected after treatment with JA or 9-Oxo-C_9 in combination with ACC (Figure 2b).

Lipid oxidation participates in the response of plants to 9-HOT

As 9-HOT increases ROS production and constitutive ET production (or application of ACC) prevents this response, we tested whether the cellular redox environment influences 9-HOT signalling. To this end, we compared the response of wild-type seedlings to 9-HOT when applied alone or in combination with various redox-active compounds, such as reduced glutathione (GSH), glutathione disulfide (GSSG; the oxidized form of glutathione), ascorbate (a key redox buffer) and Trolox (which has lipophilic anti-oxidant activity, Fluka, http://www.sigmaaldrich.com). None of the redox-active compounds analysed caused any visible phenotypic alteration compared with seedlings grown on MS (Figure S6). Moreover, we found that the application of ascorbic acid, GSH or GSSG (100 μM) did not cause major differences in the responses to 9-HOT, such as root waving, callose deposition and ROS production (Figure 3). In contrast, the three 9-HOT responses examined were inhibited when 9-HOT was applied in combination with Trolox (50 μM) (Figure 3). As with wild-type plants, Trolox inhibited the response to 9-HOT in *ein2-5* mutants, showing that the anti-oxidant activity of Trolox is exerted in an ET signalling-independent manner (Figure 3). The fact that application of Trolox, a tocopherol analogue with lipophilic anti-oxidant activity, inhibited the responses to 9-HOT suggests that oxidative stress and lipid peroxidation may be involved in 9-HOT signalling. On the other hand, the fact that tocopherols exert their lipid-protecting action by efficiently quenching singlet oxygen and scavenging various radicals (Triantaphylidès and Havaux, 2009) suggests the participation of 9-HOT in signalling responses to these reactive molecules.

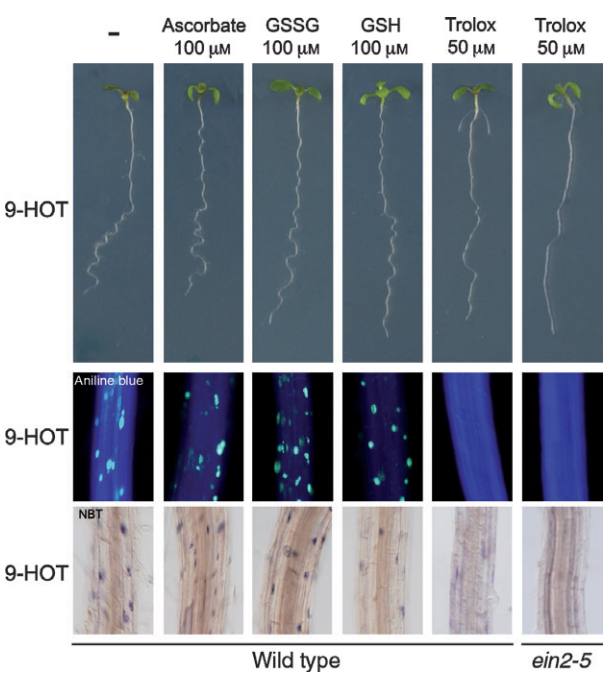


Figure 3. Effect of the redox environment on 9-HOT signalling.
(a) Phenotype of wild-type seedlings grown for 3 days in MS medium and transferred for an additional 3 days to MS medium containing redox-active compounds such as ascorbate, gluthatione disulfide (GSSG), reduced glutathione (GSH) and Trolox, in combination with 9-HOT (25 μM). Three-day-old seedlings of *ein2-5* grown in MS medium were transferred for an additional 3 days to MS medium containing Trolox (50 μM) in combination with 9-HOT (25 μM).
(b) Fluorescence visualization of callose deposition in roots stained with aniline blue (upper panels), and transmitted light visualization of superoxide anion in roots stained with nitroblue tetrazolium (lower panels).

Transcriptional changes in response to singlet oxygen varied significantly in 9-LOX and ET mutants compared to wild-type plants

Because of the involvement of lipid oxidation in 9-HOT signalling, we predicted that the response to singlet oxygen ($^1O_2$), a highly reactive oxygen species (ROS) that can initiate lipid peroxidation (Przybyla *et al.*, 2008; Triantaphylidès and Havaux, 2009), would differ between 9-HOT-deficient mutants and wild-type plants. Therefore, we examined the response of 9-LOX-deficient mutants and wild-type plants to Rose Bengal (RB), which is used to produce $^1O_2$. The *eto1-14* mutant (interfering with the 9-LOX pathway) was selected for these studies. In addition, a double mutant, *lox1-1 lox5-1* (hereafter referred to as *lox1 lox5*) containing a T-DNA insertion in each of the two 9-LOX genes of Arabidopsis, *LOX1* and *LOX5* (Bannenberg *et al.*, 2009a), was generated and included in these analyses (Figure S7). Quantification of 9-HOT to monitor 9-LOX activity revealed negligible amounts of 9-HOT in leaves and roots tissues of *lox1 lox5* seedlings. In addition, *eto1-14* seedlings showed levels of 9-HOT similar to those found in wild-type plants (Table 1).

The results from transcriptional profiles shown in Figure 4 (described in detail in Experimental Procedures) indicate that RB caused major transcriptional changes in gene expression, with 1262 genes being up-regulated and 570 genes down-regulated compared with mock-treated controls (twofold change or more false discovery rate 0.05) (Table S1). Comparison of transcriptional profiles with those obtained in previous studies using the conditional *flu*

Table 1 Levels of 9-HOT (nmol g^{-1}) in seedling homogenates incubated with linolenic acid

Genotype	Leaves	Roots
Wild-type	80.5 ± 11.5	133.2 ± 10.4
lox1 lox5	0.5 ± 0.04	0.6 ± 0.2
eto1-14	66.1 ± 1.4	102.8 ± 19.1

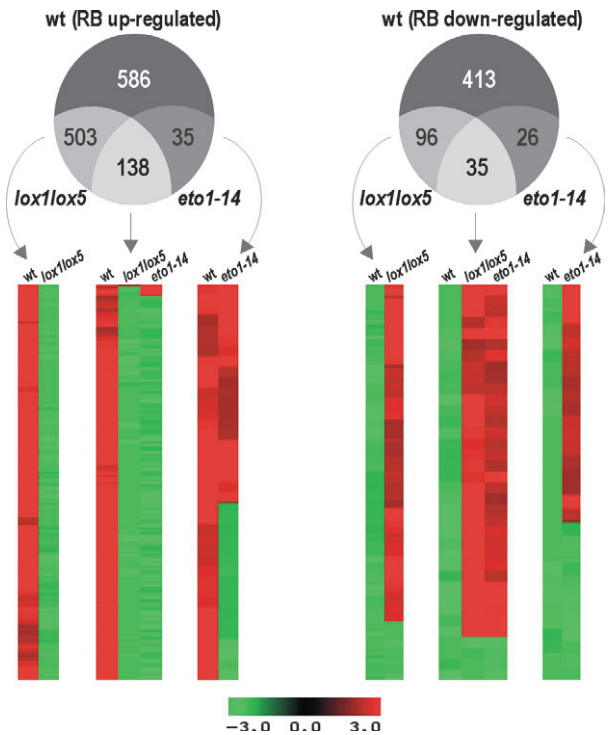


Figure 4. Microarray analyses of genes responsive to Rose Bengal (RB) in wild-type, *lox1 lox5* and *eto1-14* plants.
RNA samples were prepared from 12-day-old seedlings 3 h after Rose Bengal treatment (10 μM). Genes with twofold or greater differential expression that were either up-regulated or down-regulated in the plants examined were analysed. Venn diagrams and hierarchical clustering of genes that were up-regulated (left) and down-regulated (right) in wild-type plants after RB treatment, and of genes whose expression varied between RB-treated *lox1 lox5* and *eto1-14* mutants and RB-treated wild-type plants. Three sub-clusters are shown, representing genes whose expression varied in wild-type and *lox1 lox5* (left), in wild-type and *eto1-14* (right), or in wild-type, *lox1 lox5* and *eto1-14* simultaneously (middle). Colours indicate fold change values of differentially expressed genes on a scale of −3.0 to 3.0. A complete list of genes is given in Table S1.

mutant, which generates singlet oxygen (op den Camp *et al.*, 2003; Gadjev *et al.*, 2006), revealed that, despite important experimental differences (light-/dark-grown seedlings and non-localized production of singlet oxygen after RB treatment versus chloroplastic production of singlet oxygen in leaves of mature *flu* plants after a dark/light shift), almost 60% of the genes that showed increased expression in the *flu* mutant were also induced in wild-type plants after RB (Figure S8). Moreover, in our microarray, we found induction of genes that are known to be selectively up-regulated by singlet oxygen, but not by superoxide or hydrogen peroxide, indicating that the singlet oxygen generated by RB had specific signal activity rather than cytotoxic activity (op den Camp *et al.*, 2003; Gadjev *et al.*, 2006; Przybyla *et al.*, 2008).

Gene expression after RB treatment varied greatly in *lox1 lox5* and *eto1-14* mutants compared with wild-type seedlings. Of the RB up-regulated genes in wild-type plants, 51 and 12% showed at least twofold reduced expression in the *lox1 lox5* and *eto1-14* mutants, respectively, and only 22 genes showed increased expression in *eto1-14* plants. Similar figures were found for the genes down-regulated after RB treatment in wild-type plants, as 20 and 9% of these genes showed higher expression (two-fold or more) in RB-responding *lox1 lox5* and *eto1-14* mutants, respectively, while 3 and 2% of the genes showed reduced expression in *lox1 lox5* and *eto1-14* mutants in comparison to wild-type plants. We found that expression of 9% of the RB-responsive genes in wild-type plants (RB-induced and RB-repressed) was simultaneously modified in *lox1 lox5* and *eto1-14* mutants (Figure 4 and Table S2). Moreover, 74% of the genes that showed aletered expression in the *eto1-14* mutant compared to wild-type plants were also altered in *lox1 lox5* mutants, indicating that ET preferentially affects a subset of the transcriptional response mediated by 9-LOX activity.

Further examination of our microarrays showed transcriptional changes in *lox1 lox5* and *eto1-14* mutants that were not detected in RB-responding wild-type plants (Table S3). Thus, the expression changes of 1439 genes in *lox1 lox5* and 140 genes in *eto1-14* were unique to these mutants. Furthermore, an additional group of 203 genes was found to show altered expression in both *lox1 lox5* and *eto1-14* mutants, but not in RB-treated wild-type plants.

The possible biological function of the RB-responding genes was assessed by Gene Ontology (GO) term enrichment (Table S4). Among RB up-regulated genes, we found a significant overrepresentation of genes associated with various types of stress, regulation of gene expression, the metabolism of oxylipins and ET signalling. Terms associated with cell-wall organization and lipid metabolism were overrepresented in the pool of RB down-regulated genes in wild-type plants. Moreover, among genes whose expression varied in mutants but not in wild-type plants, we found an enrichment of terms associated with abiotic stress and photosynthesis among RB up-regulated *lox1 lox5* genes, and with amino acid, phenylpropanoid and flavonoid metabolic processes among *eto1-14* up-regulated genes.

Lack of 9-LOX activity and constitutive ET production enhance the susceptibility of plants to singlet oxygen

The altered gene expression in *lox1 lox5* and *eto1-14* mutants after RB production was indicative of an impaired response to singlet oxygen. The significance of this variation was evaluated by measuring ion leakage (as an indicator of cellular damage) in the leaves of adult plants subjected to RB treatment, as well as by examining the phenotype of seedlings when grown in the presence of RB. These analyses revealed an increase in ion leakage in *lox1 lox5* and *eto1-14* mutants compared to wild-type plants (Figure 5). Thus, the approximately 1.5-fold increase in ion leakage measured in wild-type plants 24 h after RB treatment increased

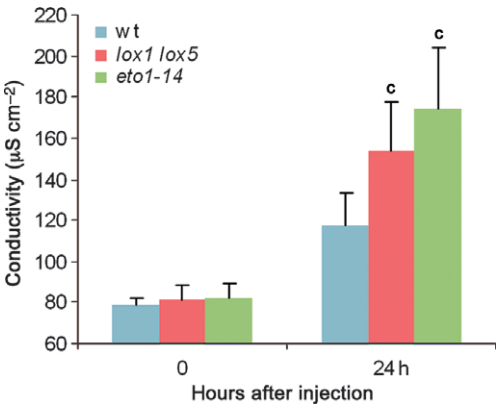


Figure 5. Measurement of ion leakage.
Conductivity measured in full-grown leaves of wild-type, *lox1 lox5* and *eto1-14* plants after injection with Rose Bengal (2 μM). Values are means and standard errors from three independent experiments are shown. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $0.01 < P < 0.05$).

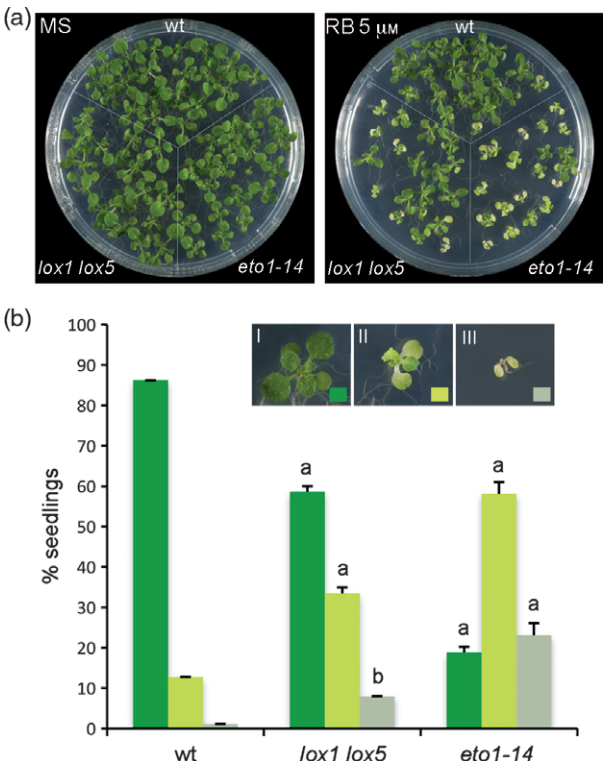


Figure 6. Analyses of phenotypic alterations in wild-type, *lox1 lox5* and *eto1-14* plants grown in RB-containing medium.
(a) Phenotype of seedlings grown for 15 days in MS medium (left) or in MS medium containing RB (5 μM) (right).
(b) Phenotypic alterations were scored on a three-point scale (I, II and III), according to the severity of the symptoms. The percentages of seedlings showing these phenotypes after 15 days of growth in RB-containing medium are shown. Values are means and standard errors from three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $^{a}P < 0.001$, $^{b}0.001 < P < 0.01$).

approximately twofold above basal levels in *lox1 lox5* and *eto1-14* mutants. Furthermore, we observed significant phenotypic variations in 15-day-old seedlings grown on medium containing 5 μM RB (Figure 6a). Rating of the phenotypic symptoms on a three-point scale (I, II and III), according to their severity, revealed a high proportion of wild-type seedlings (approximately 85%) with type I phenotype (only green leaves), compared with approximately 60 and 20% of seedlings in *lox1 lox5* and *eto1-14* mutants, respectively (Figure 6b), whereas the remaining seedlings showed type II (with green and yellow leaves) and type III symptoms (only yellow leaves). These results reveal the enhanced susceptibility of these mutants to singlet oxygen. The fact that RB caused stronger damage in *eto1-14* than in *lox1 lox5* seedlings contrasted with the reduced number of transcriptional changes found in *eto1-14*, suggesting that the qualitative differences distinguishing the transcriptional response of *lox1 lox5* and *eto1-14* to RB are critical in modulating their adaptation to oxidative stress.

Lack of 9-LOX activity and constitutive ET production enhance bacterial susceptibility and malondialdehyde accumulation

Given the attenuated response of *lox1 lox5* and *eto1-14* to singlet oxygen, and that, as described above, the genes induced by singlet oxygen in wild-type plants were enriched in responses to pathogens, we tested whether the defence potential of these mutants was diminished and whether this correlated with altered ROS regulation. Analyses of the response to the biotrophic bacterial strains *Pst* DC3000 *avrRpm1* (avirulent) and *Pst* DC3000 (virulent) revealed higher bacterial growth rates in *lox1 lox5* and *eto1-14* mutants than in wild-type controls (Figure 7a). The growth of *Pst* DC3000 *avrRpm1* did not show a significant variation in *lox1 lox5*, but increased sevenfold in *eto1-14* relative to wild-type plants. Moreover, the growth of *Pst* DC3000 increased ten- and sevenfold in *lox1 lox5* and *eto1-14*, respectively, compared with controls. Further analyses of 9-HOT-responsive gene expression revealed that, compared with wild-type plants, enhanced bacterial growth was accompanied by delayed and reduced expression, which was more apparent in the case of the virulent bacteria (Figure 7b). Analysis of the symptoms caused by bacterial inoculation revealed a slightly decreased necrosis (visible by staining with trypan blue) and increased H_2O_2 accumulation (as concluded from the strong brown coloration observed by staining with 3,3′-diaminobenzidine tetrachloride) during both interactions in *lox1 lox5* and *eto1-14* mutants (Figure 7c). Finally, we measured the accumulation of malondialdehyde (MDA), a product of uncontrolled lipid oxidation (Farmer and Davoine, 2007). As shown in Figure 8(a), there was a small but

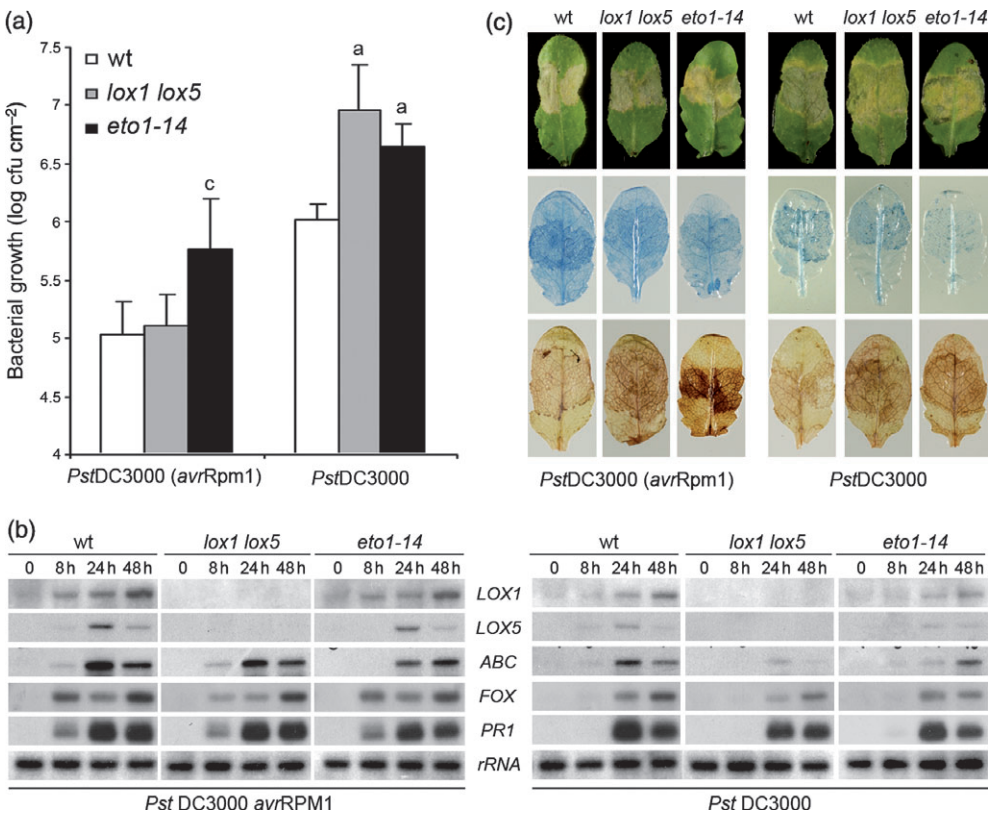


Figure 7. Response of *lox1 lox5* and *eto1-14* mutants to bacterial inoculation.
(a) Growth of *Pst* DC3000 *avrRpm1* and *Pst* DC3000 in plants 4 days after bacterial infiltration (10^5 cfu ml^{-1}). Values are means and standard errors obtained in three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $^b0.001 < P < 0.01$, $^c0.01 < P < 0.05$).
(b) Analyses of gene expression after bacterial inoculation (10^6 cfu ml^{-1}). Blots were hybridized to riboprobes for *LOX1*, *LOX5*, *ABC*, *FOX* and *PR1* genes. Hybridization against an rRNA radioactive probe was used as a loading control.
(c) Representative examples of lesions that developed in the leaves after bacterial infiltration (upper panels). Staining with trypan blue (middle panels) and 3,3′-diaminobenzidine tetrachloride (lower panels) was performed 24 and 48 h, respectively, after *Pst* DC3000 *avrRpm1* and *Pst* DC3000 inoculation.

significant increase in MDA in response to *Pst* DC3000 *avrRpm1* in *lox1 lox5* and *eto1-14* plants. A twofold increase above the levels in wild-type plants was measured in *lox1 lox5* mutants at 4 and 8 h post-inoculation, decreasing by 24 h to the levels found in wild-type plants. The MDA level in *eto1-14* mutants at 8 h post-inoculation was 1.5 times that in wild-type plants, and remained at similar levels 24 h after inoculation. Analyses of the response to *Pst* DC3000 revealed 1.8- and 1.5-fold increases in the levels of MDA in *lox1 lox5* and *eto1-14* plants, respectively, compared with wild-type plants at 8 h after bacterial infiltration.

DISCUSSION

We have previously shown that the 9-LOX product 9-HOT regulates stress responses that commonly occur during the defence of plants to pathogen infection (Vellosillo *et al.*, 2007). To obtain further insight into the molecular mechanisms involved in 9-HOT signalling, as well the role of the 9-LOX oxylipin pathway, we used a genetic approach to generate a double *lox1 lox5* Arabidopsis mutant lacking 9-LOX activity, as well as 9-HOT signalling mutants (*non-responding to oxylipins*) with an impaired response to 9-HOT. The *noxy6* and *noxy22* mutants were selected based on their insensitivity to 9-HOT (Figures S1 and S2). Identification of the *noxy6* and *noxy22* mutations as new *ctr1* (*ctr1-15*) and *eto1* (*eto1-14*) alleles, respectively, strongly indicated that enhanced ET production negatively regulates 9-HOT signalling (Figures S3 and S4). In support of this finding, we observed that application of the ET precursor ACC, in combination with 9-HOT, provoked a clear reduction of the responses to 9-HOT in wild-type plants but not in the ethylene-insensitive mutant *ein2-5* (Figure 1). Moreover, gene induction by 9-HOT reached higher levels in the *ein2-5* mutant than in wild-type plants (Figure S5), confirming a negative action of ET on the 9-HOT signalling pathway.

In addition to the inhibitory effect of ET on 9-HOT signalling, a reciprocal interference was also found, indicating that the antagonistic action of ET and 9-HOT is bi-directional. Results showing that the presence of 9-HOT reduced the ACC-induced accumulation of EIN3–GFP in

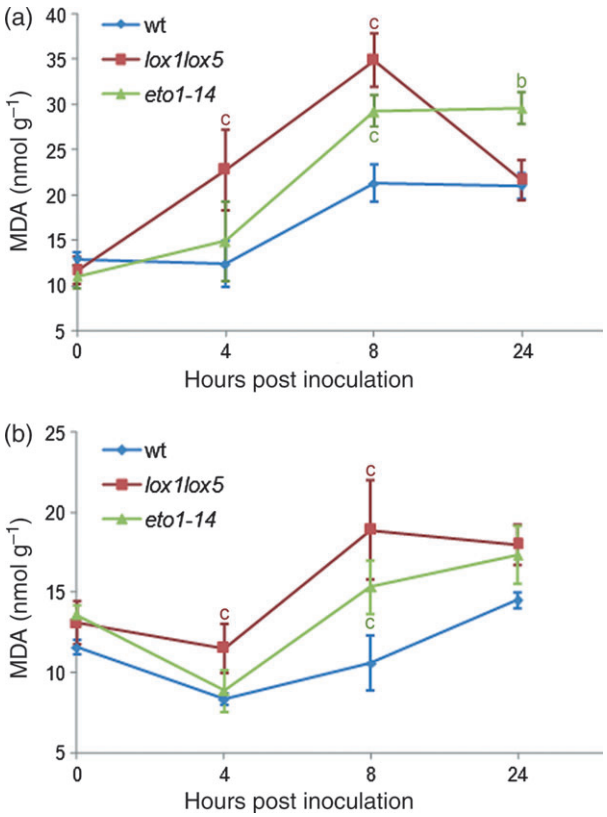


Figure 8. Analyses of MDA accumulation.
(a) Total (free and bound) MDA levels measured by GC-MS in the leaves of wild-type, *lox1 lox5* and *eto1-14* at various intervals after *Pst* DC3000 *avrRpm1* infiltration (10^7 cfu ml^{-1}).
(b) Total (free and bound) MDA levels measured by GC-MS in the leaves of wild-type, *lox1 lox5* and *eto1-14* at different intervals after *Pst* DC3000 infiltration (10^7 cfu ml^{-1}).
Values are means and standard errors from three independent experiments. Letters on top of the bars indicate statistically significant differences between the corresponding mutants and wild-type plants (Student's *t* test: $^b0.001 < P < 0.01$, $^c0.01 < P < 0.05$).

35S:EIN3-GFP transgenic plants, as well as of GUS activity in roots of *EBS:GUS* plants (Figure 2), demonstrated that 9-HOT interferes with activation of the ET pathway. Similar interference with the ET pathway was found with 9-KOT, which, like 9-HOT, induces root waving, whereas the opposite was observed with JA or 9-Oxo-C_9, which induce root growth arrest through a 9-HOT-independent signalling pathway (Figure 2 and Figure S1). The positive interaction of the JA and ET pathways was in accordance with previous results showing synergistic interaction of these hormones during the response of plants to necrotrophic pathogens (Glazebrook, 2005). On the other hand, the fact that the interaction between JA and ET differed from that of 9-HOT and ET, supported our previous data suggesting signalling diversification of these two oxylipins, JA and 9-HOT. Moreover, the fact that the action of 9-HOT and 9-KOT (9-LOX derivatives) on ET signalling differed from that of 9-Oxo-C_9 (which is also produced by the 9-LOX oxylipin pathway), supported our early results suggesting functional specialization of oxylipins according to their molecular structure rather than the specific biochemical pathway (9-LOX, 13-LOX or α-DOX) mediating their production (Vellosillo *et al.*, 2007).

The fact that application of Trolox, a tocopherol analogue with lipophilic anti-oxidant activity (Girotti, 1998), impaired the effects of 9-HOT (Figure 3), suggested involvement of oxidative stress and lipid peroxidation in the 9-HOT response. Moreover, the fact that 9-HOT enhanced ROS production was indicative of a model in which 9-HOT potentiates activation of the 9-LOX pathway in a positive feedback loop. Additionally, these results suggest participation of the 9-LOX oxylipin pathway in controlling oxidative stress and lipid peroxidation. This is supported by the results of transcriptional analyses of wild-type plants, the *lox1 lox5* mutant (lacking 9-LOX activity) and the *eto1-14* mutant (disrupting 9-HOT signalling) after application of RB, a generator of singlet oxygen with strong lipid peroxidation potential (Przybyla *et al.*, 2008; Triantaphylidès and Havaux, 2009). The fact that, of the 1832 genes responding to RB in wild-type plants, 51 and 12% showed altered expression in *lox1 lox5* and *eto1-14*, respectively, demonstrated involvement of the 9-LOX and ET pathways in the response of plants to singlet oxygen (Figure 4). In addition, the higher levels of ion leakage (an indicator of cellular damage) and increased chlorosis in tissues of RB-treated *lox1 lox5* and *eto1-14* mutants than in RB-treated wild-type plants (Figures 5 and 6) confirmed that both mutants are defective in signalling an appropriate response to singlet oxygen, and revealed their diminished ability to control this type of oxidative damage. As reported previously (Sattler *et al.*, 2004), tocopherols (vitamin E) quench singlet oxygen in a highly efficient manner, and their activity to limit lipid peroxidation is essential for germination and early plant growth. Therefore, the enhanced damage in RB-treated *lox1 lox5* and *eto1-14* mutants relative to control plants may result, at least in part, from failure to control lipid peroxidation after application of singlet oxygen.

Further studies in adult plants revealed the participation of 9-LOX and ET in the defence of plants against virulent bacteria (Figure 7). Differing actions of the 9-LOX and ET pathways in this response were deduced from the enhanced susceptibility to *Pst* DC3000 in *lox1 lox5* and *eto1-14* mutants compared with wild-type plants. The fact that the double *lox1 lox5* mutant supported approximately tenfold increased bacterial growth compared with the approximately fivefold increase found in the Arabidopsis *lox1* mutant (Hwang and Hwang, 2010, and our unpublished data, Centro Nacional de Biotecnología, Madrid, Spain) indicates the positive contribution of the two 9-LOX genes, *LOX1* and *LOX5*, to plant defence. In line with these results, the enhanced suscepti-

bility of the *eto1-14* plants could be, at least in part, a consequence of the antagonistic action of ET on 9-HOT signalling, and is in agreement with a study by Chen *et al.* (2009) showing that constitutive ET production decreased defence gene expression. In addition, recent reports revealed a positive role for ET as a regulator of pathogen-associated molecular pattern-triggered immunity (Boutrot *et al.*, 2010; Mersmann *et al.*, 2010), indicating that ET may exert different actions in response to pathogens that are operative at various layers of defence.

The results showing increased H_2O_2 accumulation in *lox1 lox5* and *eto1-14* mutants after bacterial inoculation compared with wild-type plants (Figure 7c) are indicative of an alteration in the regulation of ROS. Moreover, the enhanced accumulation of MDA, a product of lipid peroxidation, in *lox1 lox5* and *eto1-14* mutants after bacterial infection (Figure 8) is probably a reflection of a failure in signalling responses to highly reactive oxygen species, such as hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$). The fact that the *lox1 lox5* and *eto1-14* mutations may affect distinct types of ROS could be indicative of a broad regulatory role of the 9-LOX oxylipin and ET pathways on various ROS signalling pathways. Alternatively, the alterations observed could be a consequence of the interaction between distinct ROS signalling pathways as has been shown by Laloi *et al.* (2007). Independently of this, the data described suggest actions of the 9-LOX and ET pathways in the control of oxidative stress during the response to pathogen infection, in which the production of ROS must be tightly regulated to achieve full resistance and plant survival.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana wild-type, transgenic lines *35S:EIN3-GFP* and *EBS:GUS*, and *ein2-5* mutants were derived from ecotype Columbia (Col-0). The homozygous double insertion mutant *lox1 lox5* was generated by crossing individual mutants and identified by PCR as described in Vellosillo *et al.* (2007). For *in vitro* analyses, vernalized seeds were grown in Petri dishes containing Murashige & Skoog (MS) medium, pH 6.0, 1.5% w/v sucrose and 1.5 or 0.8% w/v agar (Bacto Agar; Becton-Dickinson, http://www.bd.com) for vertical or horizontal plates, respectively. Growth conditions were 16 h light/8 h dark at 22°C, with 250 μE m^{-2} sec^{-1} fluorescent illumination. Specific compounds were added to molten medium (50°C) at the indicated concentrations. Freshly prepared plates were always used to avoid product breakdown or instability. Phenotypes were observed 6 days after seed germination in approximately 15 independent seedlings (see Figure S9 as an example), and in at least three independent experiments. For *in planta* analyses, vernalized seeds were sown on soil, and grown in a growth chamber at 22°C and 70% relative humidity under a 14 h light/10 h dark photoperiod with 250 μE m^{-2} sec^{-1} fluorescent illumination.

Preparation of oxylipins

9(*S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-HOT) was prepared by stirring linolenic acid (120 mg) at 23°C with *Solanum lycopersicum* whole homogenate under an atmosphere of oxygen, essentially as described by Matthew *et al.* (1977). The product was subjected to open-column silicic acid chromatography to provide >95% pure 9(*S*)-hydroperoxyoctadecadienoic acid (HPOD) (70 mg). Treatment with sodium borohydride (100 mg) in methanol (10 ml) at 0°C for 30 min followed by preparative straight-phase HPLC using 2-propanol/hexane/acetic acid (2.2:97.8:0.005 v/v/v) as the mobile phase produced >99% pure 9-HOT as a colorless oil (44 mg; yield 35% from linolenic acid).

9-keto-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-KOT) was prepared by treating 9(*S*)-HPOD (125 mg) at 0°C for 5 min with a mixture of acetic anhydride (3 ml) and pyridine (2.4 ml). Water (1.5 ml) was added, and the mixture was stirred at 23°C for 60 min. Purification by straight-phase HPLC using 2-propanol/hexane/acetic acid (1.1:98.9:0.005 v/v/v) as the mobile phase afforded >97% pure 9-KOT as a white solid (91 mg; yield 77%).

9-oxononanoic acid (9-Oxo-C_9) was prepared by stirring 9,10-dihydroxyoctadecanoic acid (2 g) in acetone (100 ml), water (25 ml) and acetic acid (5 ml) with sodium periodate (4 g) at 23°C under argon for 90 min. The product was subjected to silica gel chromatography, followed by crystallization from hexane at 4°C, producing >98% pure 9-Oxo-C_9 as a white solid (0.41 g; yield 38%).

Assay of 9-LOX activity

Roots (0.2 g) or liquid nitrogen-powderized leaves (0.5 g) were homogenized using an Ultra-Turrax disperser (IKA-Werke, http://www.ika.net) at 0°C in 3 ml of 0.1 M potassium phosphate buffer, pH 6.7, containing 300 μM α-linolenic acid. The homogenates were stirred at 23°C for 20 min, and subsequently treated with 6 ml methanol containing 75 μg butylated hydroxytoluene anti-oxidant and 47.8 nmol of [17,17,18,18-$^2H_5$]-9-HOT as an internal standard. After centrifugation at 700 ***g***, oxylipins were isolated by extraction with diethyl ether. The products were derivatized by methyl esterification and trimethylsilylation and analysed by GC-MS as previously described (Hamberg *et al.*, 2003). The instrument was operated in scan mode for profiling of oxylipins and in selected ion monitoring mode for determination of levels of 9-HOT.

Histochemistry

Detection of callose and superoxide production was performed as described by Vellosillo *et al.* (2007). Production of H_2O_2 was visualized in detached leaves stained with 3,3′-diaminobenzidine tetrachloride (Sigma, http://www.sigmaaldrich.com/) as described by Thordal-Christensen *et al.* (1997) and Moreno *et al.* (2005). For cell death analyses, leaves were stained as described by Hamberg *et al.* (2003). *In situ* localization of GUS activity was performed as described by Malamy and Benfey (1997).

Protein extraction and Western blot

Six-day-old seedlings of *35S:EIN3-GFP* transgenic plants were transferred to liquid MS medium and subjected after 24 h to ACC (2 μM) treatment alone or in combination with 25 μM of the oxylipins (9-HOT, 9-KOT, JA or 9-Oxo-C_9). Protein extracts were prepared by grinding seedlings to a fine powder in liquid nitrogen, and extracted using buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM PMSF and 1× complete protease inhibitor cocktail (Roche, http://www.roche.com). Supernatants were obtained by centrifugation for 10 min at 3000 ***g***, separated by 10% SDS–PAGE, and transferred to nitrocellulose membranes by electroblotting. Immunoblot assays were performed as described by Sanz *et al.* (1998) using anti-Arabidopsis α-EIN3 antibody (Guo and Ecker,

2003) or anti-Arabidopsis α-RPT5 (19S regulator ATPase subunit Rpt5) antibody (Kwok *et al.*, 1999) as a loading control.

Plant treatments and gene expression analyses

For chemical treatment, RNA was prepared from 12-day-old seedlings grown on vertical MS-containing plates. Seedlings were covered with liquid MS medium (used as a control), or with liquid MS containing 9-HOT (25 μM) or RB (10 μM). Two square plates with 30–40 seedlings per plate were pooled for each time point examined. For studies of gene expression after bacterial inoculation, the abaxial side of rosette leaves of 4-week-old plants were injected with a bacterial suspension (10^6 cfu ml^{-1}). In all cases, tissues were frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was isolated as described by Logemann *et al.* (1987). RNA gel blots were performed as described by Sambrook and Russell (2001), transferred to Hybond N membranes (GE Healthcare, http://www.gehealthcare.com), and hybridized to single-stranded riboprobes according to standard procedures. In all cases, gene expression was analysed in at least three independent experiments. Radioactive probes were prepared for *LOX1* (At1g55020), *LOX5* (At3g22400), *POX* (At5g22140), *ABC* (At1g15520), *FOX* (At1g26380) and *PR1* (At2g14160). The amount of loaded RNA was verified by addition of ethidium bromide to the samples and photography under UV light after electrophoresis, followed by hybridization to an rRNA as described by Vellosillo *et al.* (2007).

Microarray hybridization and analysis

For microarray analyses, RNA was extracted 3 h after treatment and purified using an RNeasy mini kit (Qiagen, http://www.qiagen.com/). RNA was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technology Inc., http://www.nanodrop.com), and its quality was assessed using an Agilent 2100 bioanalyser (Agilent Technologies, http://www.agilent.com). RNA from four independent biological replicates was prepared and independently hybridized in three independent experiments using slides of Agilent Arabidopsis oligo microarrays 4 × 44K (reference 021169). Differential expression comparisons were performed as follows: (i) MS-treated wild-type Arabidopsis seedlings versus wild-type seedlings treated with RB-containing MS medium (10 μM), (ii) RB-treated wild-type seedlings versus RB-treated *lox1 lox5* mutant seedlings, and (iii) RB-treated wild-type seedlings versus RB-treated *eto1-14* mutant seedlings. Details of hybridization and washing of microarrays are given in Appendix S1. Raw and normalized data were deposited in the Array Express data library (http://www.ebi.ac.uk/arrayexpress/) under accession numbers E-MEXP-3009, E-MEXP-3010 and E-MEXP-3011. Significantly up-regulated and down-regulated transcripts obtained for each comparison (with a false discovery rate of 5%) are listed in Tables S1–S4. The fold change representing the differential expression is listed for each probe.

Analysis of gene ontology functional term enrichment

We used the FatiGO application (Al-Shahrour *et al.*, 2004) contained in the suite of web tools Babelomics version 3.2 (Al-Shahrour *et al.*, 2006). The server at the Centro de Investigación Príncipe Felipe (Valencia, Spain) was used to obtain an overview of the Gene Ontology (GO) functional term enrichment for RB-responsive genes. Hierarchical clustering of significant terms (adjusted *P* value <0.001) was performed using Multiexperiment Viewer (MeV) software version 4.3 (http://www.tm4.org) (Saeed *et al.*, 2003).

In vivo analyses of bacterial symptoms and *in vivo* growth curves

The pathogenic bacterial strains used in this study, *Pseudomonas syringae* pv. *tomato* DC3000 and the avirulent strain *Pseudomonas* DC3000 *avrRpm1*, were grown overnight at 28°C in Petri plates containing King's medium B. Bacterial inoculation and quantification of bacterial growth were performed as described by De León *et al.* (2002). Data were statistically analysed using Student's *t* test using GraphPad PRISM version 4 (http://www.graphpad.com/prism/Prism.htm). For symptoms tests, at least 20 plants were examined after bacterial infiltration (10^6 cfu ml^{-1}) in three independent experiments. Reported data are means and standard errors of the values obtained in three independent experiments.

Measurement of ion leakage

Cell damage was assayed by measuring ion leakage as described by De León *et al.* (2002). The abaxial side of leaves of 4-week-old plants were injected with 30 μl Rose Bengal solution (2 μM). Three leaves were treated per plant. Reported data are means and standard errors of the values obtained in three independent experiments. Data were statistically analysed by Student's *t* test as described above.

Analysis of malondialdehyde by GC-MS

Leaves were infiltrated with suspensions of *Pst* DC3000 *avrRpm1* (10^7 cfu ml^{-1}) or *Pst* DC3000 (10^7 cfu ml^{-1}). Leaf tissue was harvested at 4, 8 and 24 h post-inoculation, frozen in liquid nitrogen, and stored at −80°C until analysis. The level of malondialdehyde (MDA) was measured essentially as described by Cighetti *et al.* (2002), using methyl malondialdehyde as an internal standard and gas chromatography/mass spectrometry (GC-MS). Data were statistically analysed by Student's *t* test as described above. A detailed description of the extraction and quantification procedures is given in Appendix S2.

ACKNOWLEDGEMENTS

We are grateful to J. Paz-Ares, R. Solano and C. Flors (School of Chemistry and Collaborative Optical Spectroscopy, University of Edinburgh, UK) for critical reading of the manuscript, J. Ecker (Genomic Analysis Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, USA) for *35S:EIN3-GFP* seeds and the anti-EIN3 antibodies, J. Alonso for *EBS:GUS* seeds, M. Berrocal for initial analyses of *noxy22*, R. Piqueras for help with *in vitro* plant growth, I. Poveda for expert photography, and G. Hamberg for preparation of oxylipins. Microarray analyses were performed at the genomic facilities of the Centro Nacional de Biotecnología (http://www.cnb.uam.es/~genomica/). This work was supported by grants BIO2009-09670, EUI2008-03698 and CSD2007-00057 from the Ministery of Science and Innovation).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotypic response of *noxy6* and *noxy22* to oxylipins.

Figure S2. Response of *noxy6* and *noxy22* to 9-HOT.

Figure S3. Map-based cloning of *noxy6* and *noxy22*.

Figure S4. Phenotype of dark-grown wild-type, *ctr1-15* and *eto1-14* seedlings.

Figure S5. Expression 9-HOT-responsive genes in wild-type and *ein2-5* seedlings.

Figure S6. Phenotype of wild-type seedlings grown in the presence of redox-active compounds.
Figure S7. Scheme of genomic structures of LOX1 and LOX5 and position T-DNA insertions.
Figure S8. Comparison of transcriptional profiles from RB-treated wild-type seedlings and *flu* mutants.
Figure S9. Phenotypes of wild-type seedlings grown in vertically oriented plates.
Figure S10. Calibration curve for determination of MDA.
Table S1. Genes differentially expressed after RB treatment.
Table S2. Genes responding to RB treatment whose expression is affected by the *lox1 lox5* and *eto1-14* mutations.
Table S3. Genes whose expression changed after RB treatment in *lox1 lox5* and *eto1-14* mutants.
Table S4. GO term enrichment of RB-responsive genes.
Appendix S1. Details of microarray hybridization.
Appendix S2. Details of malondehayde determination.
Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J.R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science*, **284**, 2148–2152.

Al-Shahrour, F., Díaz-Uriarte, R. and Dopazo, J. (2004) FatiGO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. *Bioinformatics*, **20**, 578–580.

Al-Shahrour, F., Minguez, P., Tárraga, J., Montaner, D., Alloza, E., Vaquerizas, J.M., Conde, L., Blaschke, C., Vera, J. and Dopazo, J. (2006) BABELOMICS: a systems biology perspective in the functional annotation of genome-scale experiments. *Nucleic Acids Res.* **34**, W472–W476.

Andersson, M.X., Hamberg, M., Kourtchenko, O., Brunnström, A., McPhail, K.L., Gerwick, W.H., Göbel, C., Feussner, I. and Ellerstrom, M. (2006) Oxylipin profiling of the hypersensitive response in *Arabidopsis thaliana*. Formation of a novel oxo-phytodienoic acid-containing galactolipid, arabidopside E. *J. Biol. Chem.* **281**, 31528–31537.

Andreou, A., Brodhun, F. and Feussner, I. (2009) Biosynthesis of oxylipins in non-mammals. *Prog. Lipid Res.* **48**, 148–170.

Bannenberg, G., Martínez, M., Hamberg, M. and Castresana, C. (2009a) Diversity of the enzymatic activity in the lipoxygenase gene family of *Arabidopsis thaliana*. *Lipids*, **44**, 85–95.

Bannenberg, G., Martínez, M., Rodríguez, M.J., López, M.A., Ponce de León, I., Hamberg, M. and Castresana, C. (2009b) Functional analysis of α-DOX2, an active α-dioxygenase critical for normal development in tomato plants. *Plant Physiol.* **151**, 1421–1432.

Boutrot, F., Segonzac, C., Chang, K.N., Qiao, H., Ecker, J.R., Zipfel, C. and Rathjen, J.P. (2010) Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. *Proc. Natl Acad. Sci. USA*, **107**, 14502–14507.

Browse, J. (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annu. Rev. Plant Biol.* **60**, 183–205.

Caldelari, D., Wang, G., Farmer, E.E. and Dong, X. (2011) Arabidopsis *lox3 lox4* double mutants are male sterile and defective in global proliferative arrest. *Plant Mol. Biol.* **75**, 25–33.

op den Camp, R.G., Przybyla, D., Ochsenbein, C. *et al.* (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *Plant Cell*, **15**, 2320–2332.

Chen, H., Xue, L., Chintamanani, S. *et al.* (2009) ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress *SALICYLIC ACID INDUCTION DEFICIENT2* expression to negatively regulate plant innate immunity in Arabidopsis. *Plant Cell*, **21**, 2527–2540.

Cighetti, G., Allevi, P., Anastasia, L., Bortone, L. and Paroni, R. (2002) Use of methyl malondialdehyde as an internal standard for malondialdehyde detection: validation by isotope-dilution gas chromatography–mass spectrometry. *Clin. Chem.* **48**, 2266–2269.

Dangl, J.L. and Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826–833.

De León, I.P., Sanz, A., Hamberg, M. and Castresana, C. (2002) Involvement of the *Arabidopsis* α-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. *Plant J.* **29**, 61–62.

Durand, T., Bultel-Ponce, V., Guy, A., Berger, S., Mueller, M.J. and Galano, J.M. (2009) New bioactive oxylipins formed by non-enzymatic free-radical-catalyzed pathways: the phytoprostanes. *Lipids*, **44**, 875–888.

Farmer, E.E. and Davoine, C. (2007) Reactive electrophile species. *Curr. Opin. Plant Biol.* **10**, 380–386.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R. and Van Breusegem, F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiol.* **141**, 436–445.

Gao, X., Starr, J., Göbel, C., Engelberth, J., Feussner, I., Tumlinson, J. and Kolomiets, M. (2008) Maize 9-lipoxygenase ZmLOX3 controls development, root-specific expression of defense genes, and resistance to root-knot nematodes. *Mol. Plant–Microbe Interact.* **21**, 98–109.

Girotti, A.W. (1998) Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J. Lipid Res.* **39**, 1529–1542.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.

Göbel, C., Feussner, I. and Rosahl, S. (2003) Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxygenases. *J. Biol. Chem.* **278**, 52834–52840.

Guo, H. and Ecker, J.R. (2003) Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell*, **115**, 667–677.

Guzmán, P. and Ecker, J.R. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell*, **2**, 513–523.

Hamberg, M. (2011) Stereochemistry of hydrogen removal during oxygenation of linoleic acid by singlet oxygen and synthesis of 11(*S*)-deuterium-labeled linoleic acid. *Lipids*, **46**, 201–206.

Hamberg, M., Sanz, A., Rodríguez, M.J., Calvo, A.P. and Castresana, C. (2003) Activation of the fatty acid α-dioxygenase pathway during bacterial infection of tobacco leaves. Formation of oxylipins protecting against cell death. *J. Biol. Chem.* **278**, 51796–51805.

Hamberg, M., Ponce de León, I., Rodríguez, M.J. and Castresana, C. (2005) α-dioxygenases. *Biochem. Biophys. Res. Commun.* **338**, 169–174.

Hwang, I.S. and Hwang, B.K. (2010) The pepper 9-lipoxygenase gene *CaLOX1* functions in defense and cell death responses to microbial pathogens. *Plant Physiol.* **152**, 948–967.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. *Cell*, **72**, 427–441.

Kishimoto, K., Matsui, K., Ozawa, R. and Takabayashi, J. (2008) Direct fungicidal activities of C6-aldehydes are important constituents for defense responses in Arabidopsis against *Botrytis cinerea*. *Phytochemistry*, **69**, 2127–2132.

Koo, A.J. and Howe, G.A. (2009) The wound hormone jasmonate. *Phytochemistry*, **70**, 1571–1580.

Kwok, S.F., Staub, J.M. and Deng, X.W. (1999) Characterization of two subunits of Arabidopsis 19S proteasome regulatory complex and its possible interaction with the COP9 complex. *J. Mol. Biol.* **285**, 85–95.

Laloi, C., Stachowiak, M., Pers-Kamczyc, E., Warzych, E., Murgia, I. and Apel, K. (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **104**, 672–677.

Loeffler, C., Berger, S., Guy, A., Durand, T., Bringmann, G., Dreyer, M., von Rad, U., Durner, J. and Mueller, M.J. (2005) B1-phytoprostanes trigger plant defense and detoxification responses. *Plant Physiol.* **137**, 328–340.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16–20.

López, M.A., Bannenberg, G. and Castresana, C. (2008) Controlling hormone signaling is a plant and pathogen challenge for growth and survival. *Curr. Opin. Plant Biol.* **11**, 420–427.

Malamy, J.E. and Benfey, P.N. (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development*, **124**, 33–44.

Mandaokar, A. and Browse, J. (2009) MYB108 acts together with MYB24 to regulate jasmonate-mediated stamen maturation in Arabidopsis. *Plant Physiol.* **149**, 851–862.

Matthew, J.A., Chan, H.W. and Galliard, T. (1977) A simple method for the preparation of pure 9-D-hydroperoxide of linoleic acid and methyl linoleate based on the positional specificity of lipoxygenase in tomato fruit. *Lipids*, **12**, 324–326.

Mersmann, S., Bourdais, G., Rietz, S. and Robatzek, S. (2010) Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. *Plant Physiol.* **154**, 391–400.

Montillet, J.L., Chamnongpol, S., Rustérucci, C., Dat, J., van de Cotte, B., Agnel, J.P., Battesti, C., Inzé, D., Van Breusegem, F. and Triantaphylidès, C. (2005) Fatty acid hydroperoxides and H_2O_2 in the execution of hypersensitive cell death in tobacco leaves. *Plant Physiol.* **138**, 1516–1526.

Moreno, J.I., Martín, R. and Castresana, C. (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. *Plant J.* **41**, 451–463.

Mosblech, A., Feussner, I. and Heilmann, I. (2009) Oxylipins: structurally diverse metabolites from fatty acid oxidation. *Plant Physiol. Biochem.* **47**, 511–517.

Mueller, M.J. and Berger, S. (2009) Reactive electrophilic oxylipins: pattern recognition and signalling. *Phytochemistry*, **70**, 1511–1521.

Mueller, S., Hilbert, B., Dueckershoff, K., Roitsch, T., Krischke, M., Mueller, M.J. and Berger, S. (2008) General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in Arabidopsis. *Plant Cell*, **20**, 768–785.

Obregón, P., Martín, R., Sanz, A. and Castresana, C. (2001) Activation of defence-related genes during senescence: a correlation between gene expression and cellular damage. *Plant Mol. Biol.* **46**, 67–77.

Onkokesung, N., Gális, I., von Dahl, C.C., Matsuoka, K., Saluz, H.P. and Baldwin, I.T. (2010) Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in *Nicotiana attenuata* leaves. *Plant Physiol.* **153**, 785–798.

Prost, I., Dhondt, S., Rothe, G. *et al.* (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. *Plant Physiol.* **139**, 1902–1913.

Przybyla, D., Göbel, C., Imboden, A., Hamberg, M., Feussner, I. and Apel, K. (2008) Enzymatic, but not non-enzymatic, $^1O_2$-mediated peroxidation of polyunsaturated fatty acids forms part of the EXECUTER1-dependent stress response program in the *flu* mutant of *Arabidopsis thaliana*. *Plant J.* **54**, 236–248.

Rancé, I., Fournier, J. and Esquerré-Tugayé, M.T. (1998) The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. *Proc. Natl Acad. Sci. USA*, **95**, 6554–6559.

Rustérucci, C., Montillet, J.L., Agnel, J.P. *et al.* (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. *J. Biol. Chem.* **274**, 36446–36455.

Saeed, A.I., Sharov, V., White, J. *et al.* (2003) TM4: a free, open-source system for microarray data management and analysis. *BioTechniques*, **34**, 374–378.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanz, A., Moreno, J.I. and Castresana, C. (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. *Plant Cell*, **10**, 1523–1537.

Sattler, S.E., Gilliland, L.U., Magallanes-Lundback, M., Pollard, M. and DellaPenna, D. (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *Plant Cell*, **16**, 1419–1432.

Savchenko, T., Walley, J.W., Chehab, E.W., Xiao, Y., Kaspi, R., Pye, M.F., Mohamed, M.E., Lazarus, C.M., Bostock, R.M. and Dehesh, K. (2010) Arachidonic acid: an evolutionarily conserved signaling molecule modulates plant stress signaling networks. *Plant Cell*, **22**, 3193–3205.

Stepanova, A.N., Yun, J., Likhacheva, A.V. and Alonso, J.M. (2007) Multilevel interactions between ethylene and auxin in Arabidopsis roots. *Plant Cell*, **19**, 2169–2185.

Stintzi, A., Weber, H., Reymond, P., Browse, J. and Farmer, E.E. (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proc. Natl Acad. Sci. USA*, **98**, 12837–12842.

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant J.* **11**, 1187–1194.

Triantaphylidès, C. and Havaux, M. (2009) Singlet oxygen in plants: production, detoxification and signaling. *Trends Plant Sci.* **14**, 219–228.

Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C. and Grant, M. (2007) Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc. Natl Acad. Sci. USA*, **104**, 1075–1080.

Vellosillo, T., Martínez, M., López, M.A., Vicente, J., Cascón, T., Dolan, L., Hamberg, M. and Castresana, C. (2007) Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. *Plant Cell*, **19**, 831–846.

Vollenweider, S., Weber, H., Stolz, S., Chételat, A. and Farmer, E.E. (2000) Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased Arabidopsis leaves. *Plant J.* **24**, 467–476.

Wang, K.L., Yoshida, H., Lurin, C. and Ecker, J.R. (2004) Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. *Nature*, **428**, 945–950.